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RIFT VALLEY FEVER VIRUS IN MICE. V. THE PROPERTIES OF A HAEMAGGLUTININ PRESENT IN INFECTIVE SERUM

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HAEMAGGLUTININS occur in association with most viruses of the arthropod-borne encephalitides group (Casals and Brown, 1954 ; Clarke and Theiler, 1956). There are, however, few places in the world where Rift Valley Fever virus (R.V.F.) studies are permissible, except in Africa, the country of origin. No attempts to obtain haemagglutination with this virus have as yet been reported. It was thought probable that a haemagglutinin existed, for it was shown by Mims (1956) that in mice sick following R.V.F. virus inoculation, virus is bound to the red blood corpuscles, and cannot be washed off. The work reported below was therefore undertaken, and an R.V.F. haemagglutinin demonstrated.

MATERIALS AND METHODS

Virus.—A strain isolated from pooled *Eretmapodites* mosquitoes in Bwamba County, Western Uganda (Smithburn, Haddow and Gillett, 1948) was used, which had had about 150 mouse passages. Stock virus for experiments was obtained in the form of pooled serum from sick or newly dead mice which had been inoculated intracerebrally with 10^{-6} diluted infective serum. It is known that virus harvested following such small inocula is of very high titre ($10^{6 \cdot 5}$–$10^{9 \cdot 5}$ LD_{50}/0·03 ml.) and contains little or no incomplete, non-infective material (Mims, 1956).

Cells.—White Leghorn chicks less than 24 hr. old were bled from the heart into Alsever's solution. After storage at 4° for two days, the cells were washed thrice, and made up to a 10 per cent suspension in normal saline. Immediately before use a 0·25 per cent suspension of cells was prepared in the appropriate buffer for the test.

Acetone-ether extraction.—Infective serum was extracted as described for yellow fever (Y.F.) by Porterfield (1954). The dry residue was reconstituted with saline to give a 1/5 dilution of the original serum volume, and this was considered as 1/5 diluted haemagglutinin in titrations.

Haemagglutinin titrations

Serial twofold dilutions of the material to be tested were made in 0·5 ml. quantities in the appropriate phosphate buffer (0·03 M phosphate with 0·15 M-NaCl) in round-bottomed test tubes. To each tube 0·5 ml. of a 0·25 per cent suspension of red cells was added. The tubes

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were then shaken, allowed to settle for 2 hr. at the appropriate temperature, and the haemagglutination pattern read. No haemagglutination was recorded as 0, and complete haemagglutination as ++, slight agglutination as ±, and a greater degree of reaction +. The titre was expressed as the reciprocal of the dilution which gave a haemagglutination pattern of + or ++; this 0·5 ml. dilution contains one HA unit.

Haemagglutination inhibition tests

Non-specific inhibitor was removed from the serum to be tested for antibody by Seitz filtration as described by Casals and Brown (1954), serum being diluted tenfold in pH 7·0 buffered diluent (0·1 M phosphate with 0·15 M-NaCl) before filtration. The filtered serum was then diluted in twofold steps in 0·25 ml. volumes in pH 9·0 borate buffer (0·05 M borate with 0·12 M-NaCl (Clarke and Casals, 1955)). At this pH, the haemagglutinin is stable during the period of incubation. About six HA units contained in 0·25 ml. were then added to each serum dilution, and after the serum-haemagglutinin mixture had been incubated for 16 hr. at 4°, 0·5 ml. of 0·25 per cent red cell suspension in virus-adjusting diluent was added to each tube. The virus-adjusting diluent, containing 0·2 M phosphate and 0·15 M-NaCl (Clarke and Casals, 1955), brought the mixture to a final pH of 6·5, at which haemagglutination could occur. The tubes were read after standing for 2 hr. at room temperature. The inhibition titre was expressed as the reciprocal of the first dilution at which a haemagglutinin reading of ++ occurred.

Infectivity titrations.—Serial tenfold dilutions of infective material were made in 0·75 per cent bovine plasma albumin buffered to pH 7·4 and each dilution inoculated (0·03 ml.) intracerebrally to 5 or 6 mice. Mice were observed for 5 days, and end-points then calculated from deaths by the method of Reed and Muench (1938). Deaths which occurred on the first day were not included in calculations. Titres are expressed in $\log_{10}$ LD_{50}/0·03 ml.

RESULTS

Preparation and properties of the acetone-ether extracted haemagglutinin (AEHA)

Infective serum was obtained and acetone-ether extracted as described in Materials and Methods. The extract was then titrated under various pH and temperature conditions. Results (Table I) show that haemagglutination was optimal at pH 6·5, with a sharply demarcated upper pH limit at 6·8 above which there was no haemagglutination. Equally high titres occurred at 25° and at 37°, but the

TABLE I.—*Acetone-ether Extracted Haemagglutinin Titres at Different Temperatures and pH*

	pH.				
Temperature.	5·6.	6·0.	6·5.	7·0.	7·6.
4°	0	0	80	0	0
25°	40	640	1280	0	0
37°	0	320	1280	0	0

	pH.								
Temperature.	5·0.	5·3.	5·6.	5·9.	6·2.	6·5.	6·7.	6·8.	7·0.
25°	0	200	400	800	1600	1600	800	100	0

TABLE II.—*Adsorption of Acetone-ether Extracted Haemagglutinin to Red Cells*

		Time.				
		50 sec.	4 min.	8 min.	15 min.	30 min.
HA titre of supernatant	Expt. using 1 per cent cells	4	4	4	4	1
	Expt. using 5 per cent cells	4	2	0	0	0

haemagglutination pattern was clearest at 25°. Routine titrations were therefore carried out at pH 6·5 and 25°.

From its general biological properties, yellow fever virus is the virus most closely related to R.V.F., although no antigenic overlap is demonstrable either by neutralisation tests or by cross-immunity experiments. Broader antigenic relationships are shown in haemagglutination inhibition tests (Casals and Brown, 1954), and therefore these tests were done, using R.V.F. and Y.F. hyperimmune rabbit sera, together with sera from naturally infected or vaccinated human beings. It was found that although R.V.F. haemagglutination was specifically inhibited by R.V.F. immune sera, there was no inhibition by Y.F. immune sera. In another HA inhibition test it was shown that rabbit serum hyperimmune to Semliki forest virus also did not inhibit R.V.F. haemagglutination.

The purpose of acetone-ether extraction of infective serum, or of Seitz filtration of serum for antibody tests, is to remove non-specific HA inhibitors. Normal sera from three species were titrated for inhibitor by mixing 8 HA units to serial twofold dilutions of the sera before adding red cells. Results (Table III) show that

TABLE III.—*Non-specific Inhibitor Titre in Normal Sera.*

Type of serum.	Lowest serum dilution giving ++ reading.
Human (MCW) . . .	1/640
„ (McCl) . . .	1/80
Rabbit	1/320
Mouse pool A . . .	1/160
„ „ B . . .	1/80

the non-specific inhibitor titre of several normal human, mouse and rabbit sera ranged from 80–640.

Preparation of haemagglutinin from untreated infective serum

The AEHA was tested for infectivity by inoculating it undiluted intracerebrally and intravenously in adult mice. No inoculated mice died, and those receiving 0·5 ml. intravenously had not acquired neutralising antibodies to R.V.F. when tested one month later. Three mice, each of which received two 0·5 ml. intravenous inoculations of another AEHA preparation with an interval of 10 days between the inoculations, had acquired neutralising antibodies when tested one month later. Thus the AEHA is non-infective although it is antigenic. Ether is known to inactivate arthropod-borne viruses, including Y.F. and R.V.F. (Andrewes and Horstmann, 1949) and it was thought that a chemical treatment drastic enough to destroy infectivity might also have modified the properties of the haemagglutinin.

Attempts were therefore made to remove non-specific inhibitor and obtain a haemagglutinin by less drastic methods. Seitz filtration was unsuccessful, because no haemagglutinin appeared in the filtrate when tenfold diluted infective serum was passed twice through a Seitz pad. It was then shown that such filtration reduced the infectivity titre by $10^{6.0}$, and it was concluded that haemagglutinin had been removed in the same way. Centrifugation was likewise unsuccessful, for when infective serum was centrifuged at 7,000 *g* for 40 min., there was only a slight concentration of haemagglutinating material in the lower portion of the

tube. Modified chemical treatment, for instance by carrying out a single acetone + ether treatment, gave lower HA titres than did the full acetone-ether procedure. Finally, it was noticed that, whereas fresh infective serum either did not haemagglutinate or gave a zone phenomenon, serum stored at — 24° always haemagglutinated satisfactorily. An experiment was therefore performed to see whether the inhibitor in infective serum disappeared on storage at this temperature. First, it was demonstrated that heating at 56° for 1 hr. destroyed all detectable haemagglutinin in stored infective serum, but left the inhibitor titre in normal mouse serum unaltered. Thus, in the experiment, inhibitor could be titrated in infective serum, and the inhibitor titration was made more sensitive by adding only 2–3 HA units, instead of the usual 6–8 units, to each serum dilution. A large pool of fresh infective serum was obtained, and the HA and HA-inhibitor titres determined. The serum was then stored in aliquots in cotton-wool stoppered test tubes at — 24°, and at intervals an aliquot was removed and HA and HA-inhibitor titrations carried out. The results (Table IV) show that there was a

TABLE IV.—*Destruction of HA Inhibitor on Storage of Fresh Infective Serum at —20°*

	Time in hours.				
	0.	8.	23.	48.	72.
HA inhibitor titre* .	3200	1280	1600	100	<2
HA titre . . .	0	8000	4000	4000	4000

* 2–3 HA units in each tube

fall in inhibitor titre until, after three days, there was none detectable, even when twofold diluted serum was tested. The fresh infective serum did not haemagglutinate at any dilution, but after 8 hr. the amount of inhibitor had fallen sufficiently for the full HA titre to appear. It was shown on another occasion that normal mouse serum inhibitor was unaffected by a month's storage at — 24°. In further experiments, the fall in inhibitor titre of infective serum on storage was confirmed, and although it did not necessarily reach zero, it was never greater than 40, when 2–3 HA units were used. Similar small amounts of inhibitor were also detected in the acetone-ether extracted haemagglutinin. The HA titre in infective serum stored at — 24° was subsequently shown to remain steady for at least one month. Thenceforth, untreated infective serum stored under these conditions for 3 or more days was used as haemagglutinin.

It should be noted that although all haemagglutinin was destroyed by heating infective serum at 56° for 1 hr., the infective property itself was much more stable. In three separate experiments, the titre of serum treated for 1 hr. at 56° fell by $10^{0 \cdot 3}$, $10^{1 \cdot 5}$ and $10^{2 \cdot 1}$. Even after 3 hr., the titres were still $10^{5 \cdot 0}$ in both experiments in which titrations were made. Such stability at 56° was unexpected for it is often assumed that when arthropod-borne viruses are heated for 30 min. at this temperature, infectivity is destroyed.

Differences between untreated haemagglutinin (UHA) and acetone-ether extracted haemagglutinin (AEHA)

The properties of the untreated haemagglutinin were found to differ in many ways from those of the AEHA.

Titre.—The UHA had titres about 4-fold higher than did AEHA preparations ; of 16 different UHAs and 17 different AEHAs which were tested, the median titres were 3200 and 800, respectively. On one occasion a given UHA of titre 2000 was acetone-ether extracted, and the titre was then found to be 400.

pH and temperature range.—The result of a typical experiment is shown in Table V, which should be compared with Table I. Both haemagglutinins gave

TABLE V.—*Haemagglutinin Titres for UHA at Different Temperatures and pH*

	pH.			
Temperature.	5·6.	6·2.	6·5.	6·8.
4° .	0	160	640	>640
25° .	80	1280	5120	2560
37° .	—	1280	2560	—

highest titres at 25° and 37°. When the cells were allowed to settle at 4° titres were lower, being reduced tenfold for the AEHA, but only twofold for the UHA. For both haemagglutinins, highest titres were reached at pH 6·5. The AEHA action, however, was very greatly reduced at pH 6·8, and disappeared at pH 7·0, while that of UHA was only slightly reduced at pH 6·8, and in other experiments it was shown that agglutination occurred to a smaller extent even at pH 7·0 and 7·2.

Haemagglutination by UHA, therefore, occurs over a wider pH and temperature range. These differences are not accounted for by differences in the titres of the two haemagglutinins.

Effect of antiserum on agglutinated cells.—An AEHA was titrated in duplicate, using two rows of tubes, and the titres were seen to be identical when read after the usual 2 hr. Then to each tube in one titration row two drops of R.V.F. immune serum (reduced to pH 6·5 with dilute HCl) were added, and as a control each tube in the other row received two drops of pH 7·0 buffer (reduced to pH 6·5 with diluted HCl). All tubes were then reshaken, and when titres were re-read after a further 2 hr., it was seen that the immune serum had reduced the titre fourfold, whereas there was only a twofold fall in the titre of the control. This result was confirmed on another occasion. When exactly the same experiment using the same immune serum was done with a UHA, there was no fall in titre. It is concluded that haemagglutination by AEHA is more readily reversible by immune serum than is UHA haemagglutination.

Haemagglutinin stability.—An AEHA was reconstituted from powder to give the usual 1/5 dilution in pH 6·5 buffer, and a UHA (stored infective serum) diluted tenfold in the same buffer. Both haemagglutinins were then incubated at 4°, 25° and 37°, and titrations performed at intervals. It was found that at 25° and 37°, although the UHA titre was almost unchanged after 6 hr. and had fallen to low levels by 33 hr., the AEHA had almost disappeared by 6 hr. At 4°, however, AEHA was the more stable ; there was a twofold fall in titre after 16 hr., and haemagglutinin was still present at 3 days, whereas the UHA titre had fallen fortyfold by 16 hr. This anomalous behaviour at pH 6·5 was confirmed in a subsequent experiment, and was due to the fact that UHA, unlike AEHA, was far less stable at 4° than at the higher temperatures. A similar paradoxical behaviour at pH 6·7 was reported for dengue virus haemagglutinin by Sweet and Sabin

(1954). It was noticed that, at pH 6·5, both types of haemagglutinin were more stable when attached to red cells than when free in buffer.

When the haemagglutinins were buffered at pH 7·2 or pH 9·0, AEHA and UHA stabilities did not differ. Both, however, were more stable at pH 9·0 than at pH 7·2, for considerable quantities of each haemagglutinin were present after ten days' storage at 4° when the pH was 9·0, although at pH 7·2 none was left by this time.

It was thought that haemagglutinin might be more stable in whole serum than in the phosphate buffer used as diluent in the above experiments. This was shown to be so when UHA in the form of stored infective serum was incubated undiluted at 4°, 25° and 37°. At each temperature, UHA was more stable than when diluted tenfold in pH 7·2 buffer. It is known that R.V.F.infectivity, too, is more stable in protein-containing diluents than in simple salt solutions (Mims, unpublished).

Species range of red cells agglutinated.—Red cells were obtained, as routinely, from 5 animal species, as well as from the chick, and each red cell type was used in AEHA and UHA titrations. The results (Table VI) show that, although AEHA

TABLE VI.—*Comparison of Red Cell Species Agglutinated by UHA and AEHA*

Red cell species.	Titres. AEHA.	Titres. UHA.
Chick	400	800
Cock	0	200
Mice < 24 hr. old	0	400
Adult mice	0	50
Guinea-pig	0	200
Human group A	0	200
Sheep	0	0
Cow	0	0

agglutinates chick cells only, UHA agglutinates in addition the cells of 3 other species. The difference is clearly not attributable to differences in the titre of the two haemagglutinins. Several of the additional species of cells took more than 2 hr. to settle and give a clear haemagglutination pattern. Baby mouse cells, it may be noted, gave higher titres than those of adult mice, just as chick cells gave higher titres than cock cells. The experiment was repeated using different haemagglutinin preparations, and the result was confirmed, with slight differences in the exact extent to which the additional cell species were agglutinated by UHA.

Further experiments with UHA and AEHA

Adsorption.—It was shown that AEHA was rapidly adsorbed to red cells. In each of 5 test tubes, 5 AEHA units contained in 0·5 ml. were added to an equal volume of 5 per cent chick cells at pH 6·5. At intervals a tube was taken and the supernatant fluid titrated after light centrifugation. The experiment was repeated using 1 per cent chick cells. It can be seen (Table II) that all haemagglutinin had disappeared within 8 min. with 5 per cent cells, but that adsorption was slower when 1 per cent cells were used. The titre of a control 5 HA units maintained at pH 6·5 during the experiment was unchanged. In a similar experiment using pH 7·0 instead of pH 6·5 buffer, there was no adsorption after 30 min. The HA-cell combination therefore occurs rapidly, with a well-marked upper pH limit.

An experiment was done with UHA to see whether infectivity and haemagglutinin were adsorbed to the same extent by red cells. In each of three test tubes 1·0 ml. of a UHA dilution in pH 6·5 buffer was added to 0·5 ml. washed packed chick cells. The cells were kept in suspension and at intervals a tube was lightly centrifuged and HA and infectivity titrations performed on the supernatant fluid. It was found (Table VII) that all detectable HA had disappeared and that

TABLE VII.—*Adsorption of UHA and Infectivity by Chick Cells*

	Initial.	12 min.	20 min.	30 min.	Control at 30 min.
HA units/0·5 ml.	320	<2	<2	<2	320
Infectivity titre $\log_{10}$ LD_{50}/0·03 ml.	7·5	5·8	5·8	6·0	7·7

the infectivity titre had fallen to a comparable extent, within 12 min. There was no further fall in infectivity titre during the experiment, and no change in the HA or infectivity titre of control UHA maintained at pH 6·5. Two conclusions may be drawn ; first, UHA is adsorbed very rapidly to red cells, as is AEHA, and second, infective virus behaves similarly.

Elution.—When the tubes in AEHA or UHA titrations were reshaken as long as 7 hr. after settling, they re-settled to give titres which were never more than twofold lower. On reshaking after longer time intervals, the fall in titre on re-settling was attributable to inactivation of haemagglutinin at pH 6·5. There is therefore no evidence for elution at room temperature. When 640 UHA units were completely adsorbed by 0·5 ml. washed packed chick cells, there was no detectable release of haemagglutinin when the cells were incubated in pH 6·5 diluent at 37° for up to 4 hr.

In a series of experiments it was shown that when AEHA or UHA combined with chick cells, they were not removed by 4–6 washes in saline, for the cells continued to agglutinate when resuspended at pH 6·5. The HA-cell combination is therefore a firm one. It was then demonstrated that even when this firm HA-red cell combination has occurred, haemagglutination does not take place except at the correct pH. When the cells in a routine titration had settled, the supernatant fluid was removed from the last two tubes which gave ++ readings, and replaced by pH 7·0 buffer. There was then no haemagglutination in either tube, but when pH 6·5 buffer was replaced, readings of ++ again occurred. The result was the same with both AEHA and UHA, showing that the agglutination pattern occurs best at pH 6·5, even when the HA-red cell combination is already established.

Inhibitor destruction at 4° *and* 37°.—Fresh infective serum was stored at 4°, and HA and HA-inhibitor titrations carried out as described above in the — 24° experiment. There was no change in inhibitor titre after 7 days' storage, although it had been shown that UHA itself is stable under these conditions.

Fresh infective serum was then stored at 37°, and although there was some variation in different experiments, it was found that the inhibitor level was never more than slightly reduced after 24 hr. incubation. Haemagglutinin remained undetectable as a rule, and it was shown in another experiment that the UHA titre falls at least tenfold when stored for 24 hr. under these conditions. Incubated serum was shown to be sterile at the time when titrations were made.

AEHA titre of sera containing incomplete virus.—It was shown by Mims (1956) that when undiluted infective blood is passaged intravenously in mice, the infectivity titre of the blood obtained in successive passages falls precipitously. This is because very large amounts of non-infective, "incomplete" virus are present in low titre bloods.

It is known that "incomplete" influenza virus haemagglutinates, because successive HA titres remain high while infectivity titres fall during the serial passage of undiluted allantoic fluid (von Magnus, 1951). An experiment was performed to see whether "incomplete" R.V.F. virus haemagglutinated. An undiluted intravenous passage series was begun with stock high titre serum. Twelve mice were inoculated intravenously with 0·1 ml. of this serum, and when they were sick or recently dead, blood was taken from the thorax into a tuberculin syringe the needle of which contained a trace of heparin. The blood was pooled, titrated and injected intravenously in the mice of the next passage, and the remainder stored as serum at — 24°. After six passages, the stored sera were thawed, acetone-ether extracted, and HA titrations performed. On another occasion the experiment was repeated. The results (Table VIII) show that

TABLE VIII.—*Infectivity and HA Titres in the Course of Two Undiluted Intravenous Passage Series*

Passage No.	Series A. Infectivity titre.	Series A. HA titre.	Series B. Infectivity titre.	Series B. HA titre.
1	9·6	640	9·5	640
2	8·7	40	7·9	20
3	7·5	20	6·1	10
4	5·5	10	4·5	10
5	1·9	5	5·6	<10
6	8·5	320		

during each passage series the infectivity titre falls to a much greater extent than the HA titre. It is concluded that although most of the virus particles are ceasing to be infective, a relatively smaller number cease to haemagglutinate.

DISCUSSION

It has been shown that haemagglutination by R.V.F. virus is not inhibited by yellow fever or Semliki forest antisera, and therefore, R.V.F. is not one of the Group A or Group B viruses (Casals and Brown, 1954), for HA inhibition occurs between the members in these groups. Neither did R.V.F. immune serum inhibit haemagglutination by a number of Group A viruses (Venezuelan equine encephalomyelitis, Eastern equine encephalomyelitis, Semliki forest) and Group B viruses (Ilhéus, St. Louis, yellow fever) in tests carried out by Dr. J. Casals (personal communication). Serologically, therefore, R.V.F., like Bunyamwera and sandfly fever viruses, must be placed in the group of miscellaneous viruses, in spite of its close biological similarities to that of yellow fever.

Many of the properties of the acetone-ether prepared R.V.F. haemagglutinin are similar to those described for haemagglutinins of other arthropod-borne viruses prepared in the same way. For instance, haemagglutination occurs with a well-defined upper pH limit, the haemagglutinin is unstable at the optimum pH

for haemagglutination, and adsorption of haemagglutinin to red cell is rapid with no evidence of elution. It should be noted that the stability of both types of R.V.F. haemagglutinin is especially dependent on pH. This was also found to be the case for R.V.F. virus infectivity (Mims, 1956).

The destruction of HA inhibitor on storage of infective serum at − 24° but not at 37° or at 4° was an unexpected finding. It explains why R.V.F.-sick mice have the same serum inhibitor titre as normal mice, because although for a few hours before sacrifice the sick mice may have had high HA titres in the blood, inhibitor could not be destroyed. Nor is inhibitor destroyed at 4° and at this temperature haemagglutinin is stable for at least a week. Conceivably the destruction at − 24° is associated with the frozen state. There were often small quantities of inhibitor left, even after storage of infective serum for one month at − 24°. This residual inhibitor is only demonstrable when a small amount of haemagglutinin is used in the test, and it was shown that acetone-ether extracted material had a similar inhibitor content. This is not surprising, for it is known (Blix, 1941) that acetone-ether extraction of serum in the cold does not necessarily remove all the lipid substances which are presumably responsible for inhibition.

The untreated haemagglutinin differs in many ways from the haemagglutinin prepared in the customary way by acetone-ether extraction. It is the naturally occurring haemagglutinin, and during acetone-ether extraction its haemagglutinating power is appreciably weakened. Thus the HA titre is reduced about fourfold, the physical conditions required are narrowed and the agglutination of red cells other than those of the chick is no longer possible. Both haemagglutinins, however, are firmly adsorbed to red cells, and cannot be removed by washing. It was shown that, even when the union was established, haemagglutination did not occur except at the correct pH.

It is unlikely that the R.V.F. haemagglutinin plays an important part in the infective process, although the rapid union of virus and haemagglutinin with red cell could be similar to the attachment of virus to the susceptible cell. It is probable that R.V.F. virus grows in the liver of the mouse and there might therefore be HA inhibitors present in the liver which compete with red cells in combining with virus. No HA inhibitor, however, was detectable in a lightly centrifuged normal mouse liver suspension, when 2–3 HA units were used. Before the suspension was made the livers had been perfused with saline to remove most of the contained blood. Serum inhibitor titres are equally high in infected sick mice and in normal mice, so that there is no evidence for the destruction of serum inhibitor during infection. Burnet and Boake (1946) showed that ectromelia haemagglutinin, unlike vaccinia, agglutinated mouse red cells, and Mills and Dochez (1944) showed that pneumonia virus of mice haemagglutinin acted only on the cells of the mouse, the natural host. Although the R.V.F. UHA gives high titre agglutination with mouse red cells, it is noteworthy that the cells of cattle and sheep are not agglutinated, and these animals especially are infected in natural epidemics. It is possible, however, that a strain of virus which had been less extensively passaged in mice would have given different results.

The infectivity titre of the sera used in these experiments was about $10^{9\cdot0}$ LD_{50}/0·03 ml. (= $10^{10\cdot2}$ LD_{50}/0·5 ml.), and little or no incomplete virus was present (Mims, 1956). The median HA titre of 16 different sera was 3200 ($10^{3\cdot5}$) units/0·5 ml., so that there are about $10^{6\cdot7}$ LD_{50} associated with each HA unit. This figure may be compared with that obtained for allantoic fluid preparations

of egg-adapted influenza virus (Fazekas de St. Groth and Cairns, 1952); as long as there was no incomplete virus present, about $10^{6.3}$ ID_{50} were associated with each HA unit. By appropriate treatment, the infective and haemagglutinating properties of R.V.F. can be separated. Heating at 56° for 1 hr. destroyed all haemagglutinin, but there was a relatively small change in the infectivity titre. Again, the routine acetone-ether extraction destroyed infectivity while only slightly reducing the haemagglutinin titre. In spite of this, it is probable that the infective and haemagglutinating particles are identical, for both are adsorbed to red cells at about the same rate and to about the same extent. The chemical and physical treatments, therefore, have different effects on the haemagglutinating and infective properties of virus particles.

The experiments in which undiluted infective blood was passaged intravenously gave the expected falls in blood titres on successive passages (Mims, 1956). The AEHA titre did not fall to the same extent, and it is concluded that some, at least, of the incomplete virus particles haemagglutinated although they were no longer infective. It should be pointed out that the second passage sera in these experiments had AEHA titres of only 40 and 20, although infectivity titres were $10^{8.7}$ and $10^{7.9}$ respectively. Infectivity titres are no higher when virus is passaged tenfold diluted (Mims, 1956), and it is concluded that only low HA titres would be obtained in these circumstances.

The UHA described in this paper is clearly easier to prepare than the AEHA. Infective serum need only be stored for a few days at −24°, and it is then ready for use. Work is in progress to find out whether haemagglutinins for other arthropod-borne viruses can be prepared in a similar way.

SUMMARY

An R.V.F. haemagglutinin (AEHA) has been prepared by the standard method of acetone-ether extraction. Optimal haemagglutination occurred at 25° and at pH 6·5. There was a sharply demarcated upper pH limit.

There was no serological overlap with Semliki forest (Group A) or yellow fever (Group B) viruses demonstrable in HA inhibition tests.

The R.V.F. HA inhibitor present in infective mouse serum is destroyed on storage at − 24° for 3 days, so that serum containing an untreated natural haemagglutinin (UHA) is obtained. There is little or no inhibitor destruction at 37° and at 4°.

UHA is shown to be a more powerful haemagglutinin than AEHA. It is of higher titre, it acts over a greater pH and temperature range, and a greater variety of red cells are agglutinated. It is fully infective, whereas AEHA is non-infective.

Both types of haemagglutinin are rapidly adsorbed to red cells and cannot be washed off; there is no elution. Infective virus behaves similarly.

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OBSERVATIONS ON THE MODE OF MULTIPLICATION OF HERPES VIRUS

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STUDIES, made during the past decade, of the growth cycle of bacteriophage led to the formulation of a hypothesis of bacteriophage reproduction which postulates a breakdown of bacteriophage particles into sub-units which replicate independently and then recombine. Later, attempts were made to show that this hypothesis was applicable to the reproduction of animal viruses (Hoyle, 1948). The influenza virus was used in this work. For this virus (Henle and Henle, 1949; Hoyle, 1948, 1950), for the pneumonia virus of mice (Ginsberg and Horsfall, 1951) and for the viruses of herpes simplex (Scott, Coriell, Blank and Gray, 1953), vaccinia (Anderson, 1954), mumps (Wolff, 1955), and Western equine encephalitis (Rubin, Baluda and Hotchin, 1955) it has been reported that there is a period immediately after infection of the host cell when infective virus cannot be recovered, or can be recovered in quantities markedly smaller than those used to infect. This period has been called "dark" or "silent" and is also styled the "eclipse phase". Its existence is considered to be evidence for the translation of the virus into a non-infective form, or its breakdown into sub-units as a necessary stage in its multiplication. In fact Burnet (1955) in his definition of viruses, which, he admits, might be invalidated by later work, uses the clause "which undergo conversion into a non-infective form as a necessary step in their multiplication".

The present authors were unable to show that a non-infective phase occurred during the reproduction of the organism of psittacosis. Their evidence indicated that multiplication in that case was by binary fission (Bedson and Gostling, 1954). But that organism is now regarded as one of the order *Rickettsiales*, and a more "typical" virus, that of herpes simplex, was chosen for the work described here. The claim that there is an eclipse phase in the early hours of multiplication of this virus has been reinvestigated.

MATERIALS AND METHODS

The virus

The Nash strain of herpes simplex, originally isolated from the skin lesions of a patient suffering from generalised herpes by Professor A. W. Downie and later adapted to the guinea-pig, was used. Three days after intradermal inoculation of a guinea-pig in the hind foot pads, the animal was killed and the pads were excised and weighed. They were then cut up with scissors, ground with sand in a mortar, and made into a 10 per cent w/v suspension in 50 per cent skimmed milk in water containing penicillin G 250 i.u./ml., streptomycin sulphate 250 μg./ml. and mycostatin (E. R. Squibb and Son) 50 μg./ml. This 10 per cent suspension was stored at 4° and lost very little of its infectivity in the course of a month.

Cultures

1. *Maitland cultures.*—Ten-day old chick embryos, decapitated, eviscerated and weighed were cut up with scissors. 0·4 g. of the minced tissue was infected by suspending it in 1 ml.

of virus suspension at 4° overnight. The tissue was deposited by centrifugation and the supernatant removed. The tissue was washed with 5 ml. of saline four times to remove as much of the residual unadsorbed virus as possible. The tissue was then suspended in 10 ml. of nutrient fluid, which consisted of 20 per cent of normal rabbit serum, inactivated by heating at 56° for 30 min., in Parker's glucosol (Parker, 1938) as modified by Fulton and Armitage (1951). This suspension was distributed in 2 ml. amounts to Carrel flasks of 5 cm. external diameter closed with rubber stoppers. The flasks were incubated at 36° for the required period.

2. *Single cell suspensions.*—Three ten-day old chick embryos were decapitated, washed in saline and minced with the Craigie mincer (Craigie, 1949) using the coarsest plunger. The mince was suspended in 12 ml. of Parker's glucosol in a 6 in. × 1 in. (15 × 2·5 cm.) tube and allowed to stand on the bench for 10 min. The tissue having settled, as much as possible of the overlying fluid was removed and 9 ml. of a 0·25 per cent suspension of trypsin ("1:250" Difco) in Parker's glucosol at pH 7·6 was added to the tube, which was then put in the 37° water-bath for 10 min. The contents of the tube were sucked up and down with a 5 ml. syringe through a lumbar puncture needle, diluted with an equal volume of Parker's glucosol and poured through 100-mesh Monel wire gauze. The resulting cell suspension was centrifuged at 1000 r.p.m. in a horizontal centrifuge for 2 min. The deposit was washed in three changes of Parker's glucosol and finally re-suspended in 10 ml. of the same fluid and poured through 100-mesh Monel wire gauze again. To the suspension was added 2·5 ml. of inactivated normal rabbit serum, 0·3 ml. of penicillin G (20,000 i.u./ml.) and 0·06 ml. of streptomycin sulphate (20,000 μg./ml.). Such a suspension was found to contain between 4 and 8 million single dispersed cells per ml. This method of preparing a cell suspension is based on the technique of Pereira (1954). To infect the cells 10 ml. of cell suspension and 1 ml. of virus suspension were mixed in a hard glass Petri dish and left to stand at 4° overnight. The suspension was then transferred to two centrifuge tubes and the cells deposited by centrifugation at 1000 r.p.m. in a horizontal centrifuge for 2 min. The deposited cells were washed four times with Parker's glucosol, to remove as much of the residual unadsorbed virus as possible, and re-suspended in 10 ml. of a nutrient medium of the following composition: Inactivated normal rabbit serum 2·5 ml., chick embryo extract* 1·4 ml., penicillin G (20,000 i.u./ml.) 0·3 ml., streptomycin sulphate (20,000 μg./ml.) 0·06 ml., Parker's glucosol 8·2 ml. This suspension was distributed in 1 ml. amounts to hard glass centrifuge tubes (internal dimensions: diameter 1·3 cm., length 9·1 cm.). These were closed with caps of aluminium foil and incubated at 36° sloped at such an angle that the contained fluid covered about 5 cm. of the length of the tube wall.

Extraction of virus from culture

1. *Maitland cultures.*—The whole content of a Carrel flask, or the tissue therefrom after washing, was ground with sand in a mortar for 1 min., and the resulting virus suspension titrated fresh or after storage at − 70°.

2. *Single cell suspensions.*—The whole content of a hard glass centrifuge tube, or the cells, deposited by centrifugation, washed and re-suspended, fresh or more usually after storage at − 70°, were ground for 1½ min. in a glass homogeniser, and the resulting virus suspension titrated.

Neutralising antiserum

Serum of high neutralising titre was produced in guinea-pigs by repeated intraperitoneal injection of 10 per cent herpetic guinea-pig pad suspension. Serum was stored lyophilised, or in sealed ampoules at − 70°.

Titration techniques

1. *Guinea-pig skin.*—Serial tenfold dilutions of the virus suspensions were prepared in Parker's glucosol by the drop method and inoculated intradermally in the shaved flanks of female albino guinea-pigs in 0·2 ml. amounts. Readings were made at 48 hr. and recorded on the basis of naked eye assessment +++ through + to ± or ∓.

* Nine-day-old embryos were freed of their beaks, eyes and feet, washed in saline, minced with the coarsest and one but finest plungers mounted together in the Craigie mincer, mixed with an equal volume of Parker's glucosol, centrifuged at 3000 r.p.m. for 20 min., and the supernatant extract stored at − 70°. Before use it was cleared by centrifugation at 3000 r.p.m. for 20 min.

2. *Chorio-allantoic membrane of chick embryo.*—Serial tenfold dilutions of the virus suspension made in Parker's glucosol, containing 5 per cent inactivated normal rabbit serum, penicillin G (500 i.u/ml.) and streptomycin sulphate (100 μg./ml.), were inoculated in 0·05 ml. amounts on chorio-allantoic membranes (in false air sacs prepared in 11–12-day eggs by the technique of Scott (1948)). Four eggs were used for each dilution. After 40–48 hr. further incubation at 36° the inoculated membranes were removed, rinsed in saline and floated in 1 per cent formol saline in Petri dishes. Lesion counts were made on membranes infected with the highest dilution producing pocks, and those infected with the dilution immediately below that. The results are shown as the average of four counts with each dilution.

Observations on the titration techniques

The guinea-pig skin is relatively insensitive to herpes virus and the results of titration in it are recognised as being no more than approximations. However, as is shown by some of our findings recorded below, where large quantities of virus are being assayed, the titrations with guinea-pig skin run parallel with those of the chorio-allantoic membrane.

The accuracy of titration on the chorio-allantoic membrane has been investigated by Scott *et al.* (1953) who found that, for twenty titrations on the same virus suspension, the range of end-points was covered by one $\log_{10}$ unit. This must be borne in mind when considering our results. The disadvantages of this method of titration have been discussed by Kilbourne and Horsfall (1951), but it has proved the most satisfactory of the methods available to us.

RESULTS

Uptake of virus by chick embryo tissue or cells

In order to be able to measure the virus content of tissue or cells before incubation at 36°, and so before any part of the reproductive process had begun, tissue or cells were infected by contact with virus at 4°.

A preliminary test showed that the uptake of virus by cells and the later yield of virus from those cells were of the same order whether the cells were infected by overnight contact with virus suspension at 4° or by one hour's contact at 36°.

Two 4 ml. lots of the same single cell suspension were each mixed with 0·4 ml. of herpetic guinea-pig pad suspension. One lot was left overnight at 4° and the other for 1 hr. at 36°. Each lot was then centrifuged and the deposited cells washed four times and re-suspended to volume in nutrient fluid of the composition given in the section "Materials and Methods" under the heading "Single cell suspensions". Each lot was divided into four equal parts one of which was stored immediately at −70° and the others put in an incubator at 36°. After 3 hr. and after 24 hr., the cells of one part of each lot were deposited by centrifugation, separated from the fluid phase, re-suspended and homogenised. The homogenates were titrated on the chorio-allantoic membrane with the results shown in Table I.

Early hours of multiplication of virus

Growth curves in whole (Maitland type) culture.—In order roughly to determine the rates of growth of herpes virus in tissue culture, chick embryo mince was infected as described in the preparation of Maitland cultures and nutrient fluid was added without removing the infecting virus suspension or washing the tissue. The culture was distributed in 2·0 ml. amounts in Carrel flasks which were sealed with rubber corks and incubated at 36°. The virus content of one of these was determined immediately by titration in guinea-pig skin and the remainder were assayed for virus after various periods of incubation at 36°. The results of five such experiments

TABLE I.—*Comparison of Uptake and Subsequent Yield of Virus by Cells Infected at 4° and at 36°*

Period of incubation.	Dilution of cell homogenate.	Number of pocks per membrane. Infected at 4°.	Infected at 36°.
Nil	10^{-3}	33	33
	10^{-3}	2	1
3 hr.	10^{-2}	13	11
	10^{-3}	1	2
24 hr.	10^{-4}	18	35
	10^{-5}	6	6

are shown graphically in Fig. 1, from which it will be seen that in three of the experiments the virus titre of the cultures fell by 1 $\log_{10}$ unit in the early hours of incubation. This fall was detected at the 4th hour in one experiment but not until after that time in the other two. In two experiments there was no fall.

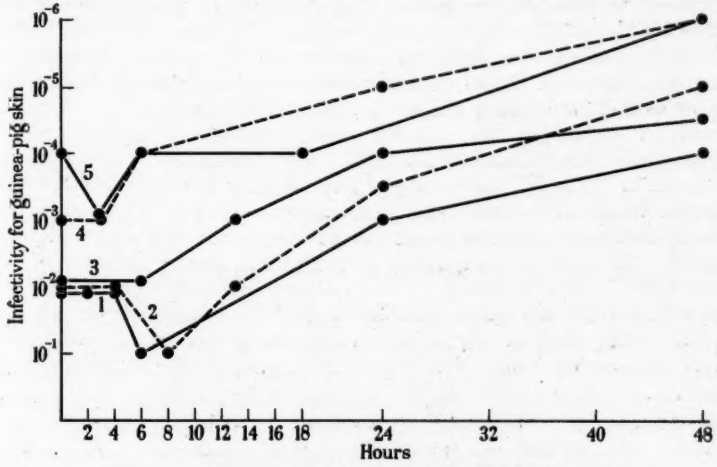

FIG. 1.—Growth curves in whole culture (Maitland-type).

Thermal stability of herpes virus.—Since in these Maitland-type cultures a considerable amount of virus probably remains extracellular, it is obvious that if this free virus is at all stable at 36° any fall in the amount of intracellular virus during the early hours of multiplication in the experiments recorded above, might have been masked by the persistence of the unabsorbed virus. The findings of Scott *et al.* (1953) suggest that herpes virus might well be stable under these conditions, and that this is so is borne out by experiments made by us to determine this point. The results of these experiments recorded in Table II show that herpes virus suspended in Parker's glucosol containing 25 per cent normal rabbit serum but in the absence of cells loses little or nothing of its infectivity at 36° over a period of 24 hr.

Growth curves obtained with washed infected tissue.—In view of this and in an attempt to avoid the confusion due to the presence of extracellular virus, cultures

TABLE II.—*Thermal Stability of Herpes Virus*

Titre.	Exp. 1. 0 hr. G.P.	Exp. 1. 0 hr. O.	Exp. 1. 24 hr. G.P.	Exp. 1. 24 hr. O.	Exp. 2. 0 hr. G.P.	Exp. 2. 0 hr. O.	Exp. 2. 24 hr. G.P.	Exp. 2. 24 hr. O.	Exp. 3. 0 hr. G.P.	Exp. 3. 0 hr. O.	Exp. 3. 24 hr. G.P.	Exp. 3. 24 hr. O.	Exp. 4. 0 hr. G.P.	Exp. 4. 0 hr. O.	Exp. 4. 24 hr. G.P.	Exp. 4. 24 hr. O.
10^{-1}	+		+		+		±		++		+		+		+	
10^{-2}	+		±		±		∓		+		±		∓		±	
10^{-3}	±		∓		∓		−		+		∓				∓	
10^{-4}	±		−		−	34	−	33	±		∓		−	58	−	47
10^{-5}	−	55	−	24	−	6	−	4	∓	55	−	44	−	4	−	10
10^{-6}	−	4	−	3	−	0	−	1	−	4	−	5	−	−	−	3

G.P. = reading in guinea-pig skin.
O. = mean pock count of four chorio-allantoic membranes.

were set up in which the chick embryo mince, after having been infected in the cold, was washed three times in amounts of Parker's glucosol which should have been sufficient to reduce extracellular virus beyond the point where it would be detectable by the chorio-allantois and, certainly, by the guinea-pig's skin. The washed infected mince was then suspended in the appropriate amount of culture fluid and distributed in Carrel flasks, virus assays being made before and after incubation at 36° for varying times. In these experiments virus titrations were made both in guinea-pig skin and on chorio-allantoic membrane. The results of the guinea-pig skin titrations in five such experiments are recorded graphically in Fig. 2, and in Table III the results of two showing the comparable findings by pock counts on the chorio-allantoic membrane are given. These show that the latter method of assay parallels the findings in the guinea-pig skin with the exception that the titres are tenfold higher owing to the greater delicacy of the pock count method, a point to which we have already alluded. The remaining three experiments showed the same parallelism but are omitted from the Table so as to avoid overloading it. Fig. 2 shows that there was no indication of loss of infectivity in the early hours of multiplication in any of the experiments (Fig. 2).

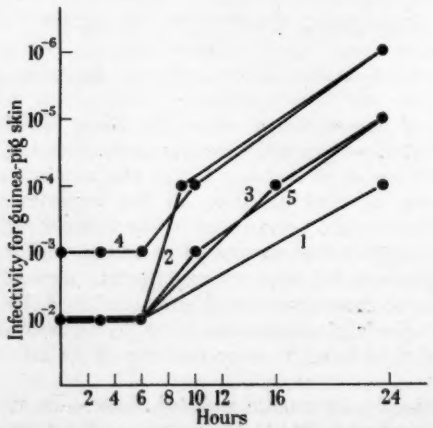

FIG. 2.—Growth curves in washed infected tissue of Maitland-type cultures.

TABLE III.—*Multiplication of Herpes Virus in Washed Infected Tissue, in Two Experiments (a and b)*

Titre.	0 hours.				3 hours.				6 hours.				9 hours.				24 hours.				48 hours.			
	G.P. skin.		Egg c.a.m.		G.P. skin.		Egg c.a.m.		G.P. skin.		Egg c.a.m.		G.P. skin.		Egg c.a.m.		G.P. skin.		Egg c.a.m.		G.P. skin.		Egg c.a.m.	
	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.	a.	b.
10^{-1}	±	±	..	..	±	±	..	..	±	±	..	..	..	++	..	..	++	+++	..	..	+++	..	..	..
10^{-2}	∓	∓	23	32	∓	∓	27	50	∓	∓	11	32	..	+	..	..	+	++	..	..	++	..	..	..
10^{-3}	–	–	1	4	–	–	1	4	–	–	0	8	..	±	..	41	±	+	..	..	+	..	..	..
10^{-4}	–	–	..	..	–	–	..	2	–	–	..	..	..	∓	..	5	∓	+	..	..	±	..	..	..
10^{-5}	–	–	..	..	–	–	..	..	–	–	..	..	..	–	..	1	–	±	50	35	∓	..	14	..
10^{-6}	–	–	..	..	–	–	..	..	–	–	..	..	..	–	..	..	–	∓	3	6	–	..	2	..
10^{-7}	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0	..	..	..	..

At this juncture it became apparent that even after washing infected mince or cells several times, further washing would continue to remove virus. Using influenza A virus, Ackermann, Ishida and Maassab (1955) have found that infected chorio-allantoic membranes washed in six changes of buffered saline and then a further nine times in culture medium at 37° for 20 min. at a time, still released virus into the washings. Clearly virus assays on homogenised washed tissue or cells will measure this reversibly attached virus as well as intracellular virus, and a fall in the level of the latter might be masked by the continued presence of the former during the early hours of multiplication.

The following experiment was made to show the effect of washing suspensions of dispersed cells infected with herpes virus :

Single cells in suspension were infected by overnight contact at 4° with herpetic guinea-pig pad suspension. The cells were then repeatedly washed with Parker's glucosol. Samples of the 4th, 8th and 12th wash and of the re-suspended cells after each of those washes were saved. Parts of the cell samples were incubated at 36° for 24 hr. The results of assays on the washes and on the cells before and after incubation are shown in Table IV. These results show that about one-tenth of the virus associated with the cells is removed at each wash, so that altogether the greater part of this virus may be reversibly attached. It can also be seen that the yield of virus from the incubated cells declines as the number of washings increases ; the change in the level of virus at the onset of incubation is insufficient to account for a fall in yield of 2 $\log_{10}$ units between the 4th and 12th washings, and it is probable that this fall is due to destruction of cells by frequent washing.

TABLE IV.—*Separation of Virus from Cells by Washing.*

Number of washes.	Dilution of titrated material.	Number of pocks per membrane.		
		Washing fluid.	Cells before incubation.	Cells after incubation.
4	10^{0}	24	—	—
	10^{-1}	5	25	—
	10^{-2}	—	1	—
	10^{-3}	—	—	66
	10^{-4}	—	—	8
8	10^{0}	6	—	—
	10^{-1}	1	14	—
	10^{-2}	—	2	51
	10^{-3}	—	—	3
12	10^{0}	5	51	—
	10^{-1}	0	10	93
	10^{-2}	—	—	3

These considerations led us to conclude that in order to be able to assay intracellular virus, reversibly attached virus would have to be neutralised with antibody. Virus within living cells is unaffected by antibody (Rous, McMaster and Hudack, 1935), and Scott *et al.* (1953) used antiserum to terminate the period of herpes virus uptake by the chorio-allantoic membrane in the egg, later washing the chorionic surface of the membrane *in situ* before excising it for virus assay. Ackermann *et al.* (1955), in the experiments with influenza virus already cited, incorporated immune serum in the fluid used to wash chorio-allantoic membranes,

and found the washed membranes contained one-tenth of the amount of virus found in control membranes washed with fluid containing no immune serum. Wildy (1954) introduced antiserum into de-embryonated eggs 1 hr. after infection with herpes virus in order to remove residual seed virus. The antiserum was removed 2 hr. after its introduction and replaced by glucose Ringer for the rest of the experiment.

In our experiments single cell suspensions were infected by contact with herpetic guinea-pig pad suspension at 4° overnight. The cells were deposited by centrifugation and the supernatant containing the unadsorbed virus of the infecting suspension was saved for assay. The cells were washed 4 times with Parker's glucosol, half the volume of cells was re-suspended in the nutrient medium specified in the section on "Materials and Methods", and half in the same nutrient medium containing anti-herpes guinea-pig serum in a concentration of 1/14. These two suspensions, constituting a control and an antibody series, were each distributed in 1 ml. amounts in 5 hard glass centrifuge tubes which were put in the refrigerator overnight. On the following morning the whole contents of one tube of each series was put in ampoules and stored at −70°. A second tube of each series was centrifuged and the culture fluid, and the cells after being twice washed with Parker's glucosol containing 5 per cent normal rabbit serum and re-suspended in that fluid, were stored in ampoules at −70° separately. The remaining three tubes of each series were transferred from the refrigerator to the 36° incubator where they were left for 3, 6 or 24 hr. before being handled like the second tubes. The results of titrations in four such experiments on the unadsorbed virus of the infecting suspension, and, in the antibody series on the homogenised unincubated whole culture and the four samples of separated washed and re-suspended cells, are shown in Table V. Table VI shows the results for the control series, in which are also included the results of titrations on the culture fluids corresponding to each sample of washed cells.

These results show that in the antibody series (Table V) sufficient antibody was present to neutralise all the virus extractable from the cells at the beginning of incubation. They also show that the uptake of virus by the cells was related directly to the titre of the infecting suspension, being a little less than 1 per cent of it; a similar relationship was observed in Maitland type cultures. The level of infective virus in the cells declined in the early hours of incubation to about one-tenth of the initial uptake and, where the uptake had been small, little or no infective virus could be detected at 6 hr. (Table V, Expt. 2 and 4). It is also clear that the extent of virus multiplication did not depend on the initial uptake. A comparison of the titrations made on the two series (Table V and VI) shows that at "0 hours" the antibody-treated cells contain about one-tenth of the amount of virus found in the control cells and that this order of difference between the two series persists throughout the period of observation. Scott *et al.* (1953) found a similar tenfold difference after 24 hr. between chorio-allantoic membranes infected with herpes virus and incubated *in ovo* in the presence of antiserum and those without, and they attributed this to residual antibody in the homogenates of the washed antibody-treated membranes. This could not explain our findings since the antiserum used when diluted 1/512 had little or no effect on the pock count given by a suspension of herpes virus diluted to produce 100 pocks, and the washings to which the cells were subjected in our experiments were calculated to dilute residual antibody beyond a concentration of 1/20,000. It would seem clear,

TABLE V.—*Culture of Virus in Chick Embryo Cells Suspended and Incubated in Nutrient Medium Containing Herpes Antibody*

Exp. No.	Dilution of tested material.	Number of pocks per membrane. Unadsorbed virus of infecting suspension.	Homogenate of unincubated whole culture.	Homogenates of cells separated, washed and re-suspended after incubation at 36° for: 0 hours.	3 hours.	6 hours.	24 hours.
1	10^{0}	—	0	—	21	18	—
	10^{-1}	—	—	27	4	2	9
	10^{-2}	—	—	5	—	—	2
	10^{-3}	74	—	—	—	—	—
	10^{-4}	6	—	—	—	—	—
2	10^{0}	—	0	6	1	0	—
	10^{-1}	—	—	1	—	—	20
	10^{-2}	16	—	—	—	—	4
	10^{-3}	2	—	—	—	—	—
3	10^{0}	—	0	—	7*	2*	†
	10^{-1}	—	—	10	1	1	—
	10^{-2}	—	—	2	—	—	37
	10^{-3}	—	—	—	—	—	5
	10^{-4}	19	—	—	—	—	—
	10^{-5}	1	—	—	—	—	—
4	10^{0}	—	0	5*	1*	0*	—
	10^{-1}	—	—	0	0	—	13
	10^{-2}	—	—	—	—	—	2
	10^{-3}	26	—	—	—	—	—
	10^{-4}	2	—	—	—	—	—

The corresponding culture fluids from which the cells had been separated were always tested on 4 membranes. In no case did they contain infective virus. † 51 hours not 24 hours. * Average of 8 membranes.

therefore, that the reduction in our experiments brought about by the treatment with antiserum of infected, washed but unincubated cells must be attributed to the neutralisation of virus which, though attached to the cells, had not reached an intracellular location, *i.e.*, reversibly-attached virus to which reference has already been made.

DISCUSSION

By infecting in the cold the cells or embryo mince with which our cultures were initiated, it is thought that a more accurate base line from which to commence plotting the growth curves has been made possible. Any prior breakdown of the herpes virus into hypothetical sub-units and replication in this form would in this way be excluded. And since, in addition to this, the cold-infected cells have been washed and then treated in the cold with herpes antiserum before setting up the cultures, the virus detectable at the beginning of incubation should represent with some accuracy the total virus content of the cells. Further, by having an amount of herpes-neutralising antibody in the fluid phase of the culture adequate to deal with any extracellular virus, which was the case in our later experiments, an uncomplicated single cycle of multiplication of the virus would be ensured. It

TABLE VI.—*Culture of Virus in Chick Embryo Cells Suspended and Incubated in Nutrient Medium without Herpes Antibody*

Exp. No.	Dilution of tested material.	Number of pocks per membrane. Unadsorbed virus of infecting suspension.	Homogenate of unincubated whole culture.	Homogenates of cells separated, washed and re-suspended after incubation at 36° for: 0 hours. Cells.	0 hours. Fluid.	3 hours. Cells.	3 hours. Fluid.	6 hours. Cells.	6 hours. Fluid.	24 hours. Cells.	24 hours. Fluid.
1	10^{0}	—	—	—	—	—	—	—	18	—	—
	10^{-1}	—	78	—	10	27	27	4	3	—	10
	10^{-2}	—	7	11	3	5	2	1	—	15	1
	10^{-3}	74	—	3	—	—	—	—	—	1	—
	10^{-4}	6	—	—	—	—	—	—	—	—	—
2	10^{0}	—	—	45	2	9	2	6	3	—	—
	10^{-1}	—	6	4	—	1	—	0	—	—	3
	10^{-2}	16	1	—	—	—	—	—	—	72	0
	10^{-3}	2	—	—	—	—	—	—	—	13	—
3	10^{0}	—	—	—	—	47	—	25	—	†	†
	10^{-1}	—	19	—	27	3	19	3	10	—	—
	10^{-2}	—	2	23	1	—	1	—	2	—	150
	10^{-3}	—	—	2	—	—	—	—	—	67	—
	10^{-4}	19	—	—	—	—	—	—	—	—	—
	10^{-5}	1	—	—	—	—	—	—	—	—	—
4	10^{0}	—	—	23	20	2	5	1	1	—	—
	10^{-1}	—	11	2	3	0	0	0	0	—	19
	10^{-2}	—	1	—	—	—	—	—	—	—	1
	10^{-3}	26	—	—	—	—	—	—	—	24	—
	10^{-4}	2	—	—	—	—	—	—	—	—	—

† Exp. 3, last samples taken at 51 hours not 24 hours.

has been found that under these cultural conditions infective virus can be detected throughout the period of virus development provided that the inoculum was not a very small one. In this respect it might be recalled that we were titrating on the chorio-allantois a strain of herpes virus which had not been maintained in passage in this tissue and it is possible that a more delicate method of titration than those available to us might have revealed virus where none was found. However, with the larger inocula the level of infective virus after 3 hr. incubation was about one-tenth of that found at the beginning, and it remained at this or a slightly lower level until 6 hr. Then, as shown by the experiments made with Maitland-type cultures (Fig. 2), the growth curves began to rise, this rise being detectable after 8 hr. The question of the origin of the new virus appearing at the 8th hour then arises. Is it derived from the 10 per cent of the original virus uptake which is still detectable as infective virus at the 6th hour of cultivation or does it come from the 90 per cent which cannot then be found? Numerous investigators, after work with this and other animal viruses, have been confronted with this question and the majority have concluded that the new virus is derived from the latter fraction. They believe that the portion of the inoculum which becomes non-infective breaks down into sub-units which are replicated by the enzyme systems of the infected cells and subsequently recombine to form the

new complete virus. The evidence in support of this conception is as follows. In the first place the results of experiments with bacteriophage are cited by analogy. During the first minutes after infection of a bacterium with a bacteriophage no biologically active phage can be recovered (Doermann, 1952) nor can bacteriophage particles with their characteristic morphology be observed with the electron microscope (Levinthal and Fisher, 1952) when the host bacteria are disrupted. There is apparently an absolute " eclipse " and it is therefore assumed that the new generations of bacteriophage arise by some method other than binary fission of existing phage particles. But in the case of herpes virus there is no absolute eclipse and in this our findings are similar to those of other workers (Scott *et al.*, 1953 ; Modi and Tobin, 1954 ; Wildy, 1954). So on this score there is no necessity for the assumption that herpes virus multiplies in the same way as bacteriophage. Then Henle and Liu (1951) have described the phenomenon of multiplicity reactivation of influenza A and B viruses which had been inactivated by ultra-violet light, a phenomenon at one time held to be evidence for the sub-unit method of multiplication of bacteriophage (Luria, 1947). It has recently been shown by Cairns (1955), however, that the results obtained by Henle and Liu could equally well be explained by the blocking of receptors by inactive virus in the inoculum with prevention of absorption of the yield of the first cycle of multiplication and so an abnormal rise of extracellular infective virus.

A third point held to be in favour of sub-unit replication is the production of hybrid recombinant forms by the simultaneous infection of the same cell by different strains of the same virus (Burnet and Lind, 1951 ; Hirst and Gottlieb, 1953). The view that the new forms, separated by passage at limiting dilutions, are, in fact hybrid recombinants and not mixtures, turns on the questionable assumption that a minimal infective dose is the same as one virus particle, although the evidence provided by Hirst and Gottlieb (1953) is not open to this objection. However, in view of the classical work of Griffith (1928) on the transformation of pneumococcal types and of the later investigations of Avery, MacLeod and McCarty (1944) of this phenomenon, one wonders whether it is necessary to postulate sub-unit replication to explain virus hybridisation.

Another piece of evidence thought to support sub-unit replication has recently been advanced by Hoyle and Frisch-Niggemeyer (1955). Making use of influenza A virus labelled with ^{32}P they have studied the distribution of ^{32}P in extracts of the chorio-allantoic membranes taken 90 min. after infection. It was found that 78 per cent of the ^{32}P could not be sedimented from such extracts by centrifugation at 100,000 *g* for 3 hr. ; in fact only 14 per cent was thrown down by centrifugation at 20,000 *g* for 1 hr., a process sufficient to deposit 98 per cent of the ^{32}P from a suspension of labelled virus. As these authors rightly point out, the unsedimentable ^{32}P must have come from disintegrated virus, and they also state that any phosphorus-containing cell fraction which can be made appears to be radioactive 90 min. after infection with labelled virus. But in view of Hoyle's (1950) postulated structure of the influenza elementary body as an aggregate of group and specific antigen particles in an ether-soluble envelope, the breakdown of the labelled virus to particles so small as to be unsedimentable at 100,000 *g* suggests a disintegration which was more complete than was expected and which had gone further than breakdown to sub-units. The fate of the 14 per cent of ^{32}P which was sedimented at 20,000 *g* is not discussed by these workers who apparently did not

consider the possibility that new virus might arise from this fraction, which is reminiscent of the 10 per cent of the initial virus uptake still detectable in the early hours of incubation in our experiments. Thus the evidence supporting the "eclipse" conception of virus multiplication seems inadequate, but if, on the other hand, new virus arises from the much smaller fraction which remains detectable throughout the early hours of cultivation, what becomes of that which one could not detect? We have shown that so much virus cannot have been lost by thermal degradation. The findings of Gray and Scott (1954) suggested to us that it might have entered the nucleus and so escaped extraction by our method of homogenisation, but work in progress in this laboratory does not support this hypothesis. It must be supposed, therefore, that either it has been destroyed by the cells or that it has become irreversibly attached to them. Hoyle and Frisch-Niggemeyer's (1955) evidence supports the former view. One might refer here to the work of Mackaness, Smith and Wells (1954) on the multiplication of the tubercle bacillus in cultures of rabbit monocytes. Direct counts of the number of bacilli in the infected monocytes were made and the proportion of all monocytes infected after 1, 3, 5, 7 and 9 days' incubation was recorded. Virulent strains multiplied steadily; the less the virulence the longer the lag before any increase occurred. In the case of B.C.G. there was an actual loss by digestion of 40 per cent of the bacilli by the 5th day and any increase in their numbers was delayed to the 10th or 12th day. Had these workers been virologists and not bacteriologists working with a micro-organism readily demonstrable with the microscope, they would probably have called the loss of bacilli before multiplication an "eclipse". These findings with the tubercle bacillus do, in fact, recall some investigations made by Davenport and Francis (1951) with strains of influenza A and B adapted and unadapted to mouse lung. The adapted strains multiplied continuously in mouse lung from the inception of infection whereas the unadapted strains showed a loss of virus at the 6th hour and subsequently multiplied to a lower titre than the adapted strains. May not the early loss in the case of the unadapted strains of influenza virus have been due to destruction of some of the virus by the cells? In our opinion, therefore, the conception of an "eclipse" in virus multiplication and all it implies remains unproven and the view that these agents multiply like bacteria by simple division must still be kept in mind.

SUMMARY

The virus content of whole Maitland-type cultures, made with chick embryo mince infected by exposure to herpes virus overnight at 4°, was titrated at the beginning of incubation at 36° and at intervals thereafter.

These experiments were repeated but the virus content of the washed tissue of the culture alone was assayed. No eclipse was found.

Similarly infected cultures of dispersed chick embryo cells in a medium containing anti-herpetic serum were then investigated. In these experiments, in which extracellular virus was neutralised, the quantity of virus detectable in the cells after 3 and 6 hours' incubation had declined to about one-tenth of its initial level, but was measurable at these times provided the infecting dose had been sufficiently large.

The possible interpretations of these results, and their bearing on the hypotheses of herpes virus multiplication processes, are discussed.

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THE XANTHINE OXIDASE GROUPS. A PHENOMENON ASSOCIATED WITH THE MULTIPLICATION OF NEUROTROPIC VIRUSES

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IT has previously been shown that a marked rise of xanthine oxidase activity occurs during the multiplication of the 17D strain of yellow fever virus in mouse brain (Bauer, 1947*a*); a similar rise was also observed with the French neurotropic strain of yellow fever, and with the viruses of lymphogranuloma venereum and lymphocytic choriomeningitis, and a rise of lesser extent occurred in chick embryos during infection with 17D (Bauer, 1947*b*, 1948). Similar experiments with neurotropic yellow fever, lymphocytic choriomeningitis, neurovaccinia and dengue viruses showed that the activities of certain other enzymes of nucleic acid metabolism were increased in mouse brain during the course of infection (Bauer, 1951), and it was further noted that the extent of the rise above the normal level of activity was not the same for each virus. It was therefore of interest to investigate the activity of these enzymes, and of xanthine oxidase in particular, in other virus infections, in order to establish whether the increase of enzyme activity is a characteristic feature of virus infections in general, and whether the extent of the rise is a characteristic property of the virus concerned.

METHODS

Assay of Xanthine Oxidase Activity

In previous work the xanthine oxidase activity of the mouse brain extracts was determined by recording the time of decolourisation of methylene blue in Thunberg tubes under standard conditions (Bauer, 1947*b*), but the method is not sensitive enough to give a reliable result with low enzyme activities, and the end-point is difficult to estimate as the concentration of unreduced methylene blue approaches zero asymptotically. Attempts to determine the activity by measuring the rate of uptake of oxygen in the Warburg apparatus (Axelrod and Elvehjem, 1941) were unsuccessful, since the enzyme activity of mouse brain extracts was usually too low for accurate measurement. A method based on the reduction of triphenyltetrazolium chloride was therefore developed; it proved to be far more sensitive than the methylene blue method and enabled the xanthine oxidase activity to be determined to three significant figures. Only the method finally adopted will be described here, but much preliminary work was done to determine the optimum conditions of extraction of enzyme, concentration of reagents, pH, use of inhibitors and time and temperature of incubation.

The following stock solutions were used: 2 : 3 : 5-triphenyltetrazolium chloride, 20 mg./ml.: this should be stored in the dark and should not be kept for longer than 1 week; hypoxanthine, 1·6 mg./ml. in 0·1 N-NaOH, adjusted to pH 9·4 with 0·1 N-HCl, and diluted 1/10 before use; the solution can be kept for 2 weeks in the refrigerator; 0·2 M pH 9·4 borate buffer, containing 0·2 M sodium pyrophosphate. Extracts of mouse brain are made by grinding by hand in a porcelain mortar with sufficient buffer to make a 10 per cent (w/v) suspension, which is clarified by low-speed centrifugation. The supernatant fluid, which contains all the xanthine oxidase activity, is removed for assay: 0·3 ml. is placed in a test tube with 0·25 ml. triphenyltetrazolium chloride solution, 0·25 ml. diluted hypoxanthine solution, 0·5 ml. borate buffer and water to bring the total volume to 2·25 ml.; the tube is

closed with a rubber bung fitted with a glass tube, rubber tube and screw clamp, and the tube is exhausted with a rotary oil pump. Brain extracts will reduce triphenyltetrazolium chloride directly, and this unspecific activity must be controlled in all cases by setting up a blank tube with 0·25 ml. water in place of hypoxanthine. During setting up and evacuation the tubes must be screened from bright daylight, which causes photochemical reduction of tetrazolium compounds; after evacuation the tubes are incubated at 37° for 60 min. The triphenyltetrazolium chloride acts as an electron acceptor in the oxidation of hypoxanthine and is thereby reduced to the insoluble red triphenylformazan. At the end of the incubation period air is readmitted, and the triphenylformazan is brought into solution by adding 5 ml. of glacial acetic acid and extracted into 5 ml. of toluene. The optical density at 495 mμ is then determined in a Unicam SP600 spectrophotometer, and the difference between the test and blank values gives a measure of the enzyme activity. Preliminary experiments showed that there was a linear relation between enzyme activity and optical density over a range of activity considerably greater than that encountered in practice. A standard curve relating optical density to amount of triphenyltetrazolium reduced, and thus to the amount of hypoxanthine oxidised, was obtained by reducing known amounts of triphenyltetrazolium chloride with ascorbic acid in 0·1 N-NaOH. The optical density of the triphenyl-formazan in the toluene extract remains constant for at least 7 days, and the extracts may therefore be kept until it is convenient to read them.

Viruses

Mice were inoculated intracerebrally with the viruses mentioned below, and brains were removed for assay at a time when signs of encephalitis were well advanced. Attempts were made to select animals at the same stage of illness, avoiding animals which were moribund or in which signs were just appearing. In most cases the viruses were maintained by intracerebral passage. The sources from which they were obtained are given in the acknowledgements at the end of the paper; when the source is not mentioned the viruses concerned were of uncertain origin and had been maintained in the laboratory for many years. In order to simplify the presentation of Figures and Tables each virus had been allotted a serial number, according to the following key:

1, *Senger*; a virus of encephalomyocarditis type isolated "from a human source" by Bieling (Findlay and Howard, 1951). 2, *Bwamba* fever; 3, *Neurovaccinia*; the IHD strain of vaccinia adapted to passage in mouse brain; 4, *Pseudolymphocytic choriomeningitis*, Sandom strain; 5, *Encephalomyocarditis*; 6, *Fantz*; a virus of encephalomyocarditis type of uncertain origin; 7, *California*, BFS 283 strain; 8, *Bunyamwera*; 9, *Neurotropic Rift Valley fever*; 10, *MM*; 11, *Mengo*; 12, *Mouse encephalomyelitis*, GD VII strain; 13, *Mouse encephalomyelitis*, TO strain; 14, *Poliomyelitis*, MEFl strain; 15, *Columbia-SK*; 16, *Influenza A*, PR8 strain; 17, *Influenza A*, NWS strain; 18, *Haemagogus A*; 19, *Anopheles B*; 20, *Herpes simplex*; 21, *Yellow fever*, French neurotropic strain; 22, *Leucocelaenus*; 23, *Sabethes*; 24, *Anopheles A*; 25, *JWS*; a recombinant between JHM and the NWS strain of influenza A (Andrewes, Isaacs and Marmion, 1954); 26, *Rabies*, Flury strain; 27, *JHM*; 28, *Wyeomyia*; 29, *Haemagogus B*; 30, *Semliki Forest*; 31, *Neuro-Mel*; a recombinant between the NWS and MEL strains of influenza A (Burnet and Lind, 1951); 32, *Neuro-Kunz*; a recombinant between the NWS strain of influenza A and the Kunz strain of A-prime (Appleby, 1952); 33, *Meningopneumonitis*, MP-F97 strain; 34, *Sandfly fever*, Naples strain; 35, *Lymphogranuloma venereum*; the JH strain adapted to passage in mouse brain; 36, *Ntaya*; 37, *Ilhéus*; 38, *Dengue type* 2, New Guinea strain; 39, *Uganda S*; 40, *Sandfly fever*, Sicilian strain; 41, *García*, a mouse encephalomyelitis virus of TO type of uncertain origin; 42, *Colorado tick fever*, Condon strain (C-1426); 43, *Neuro-Swine*; a recombinant between the NWS and Swine strains of influenza A (Burnet and Lind, 1951); 44, *Yellow fever*, 17D strain; 45, *West Nile*; 46, *Dengue type* 1, Hawaii strain; 47, *Zika*.

RESULTS AND DISCUSSION

The xanthine oxidase activities of mouse brains at the height of infection with the various viruses are shown in Fig. 1. The abscissa is the logarithm of the optical density of the triphenylformazan produced under the standard conditions of the assay. The first horizontal line represents the logarithm of the mean,

standard deviation and range of variation of the xanthine oxidase activity of normal mouse brain ; each successive horizontal line represents the same set of values for each virus in turn, the viruses being arranged along the ordinate according to their serial numbers. For normal mouse brain the arithmetic mean of the individual values was calculated. During the course of infection the xanthine oxidase

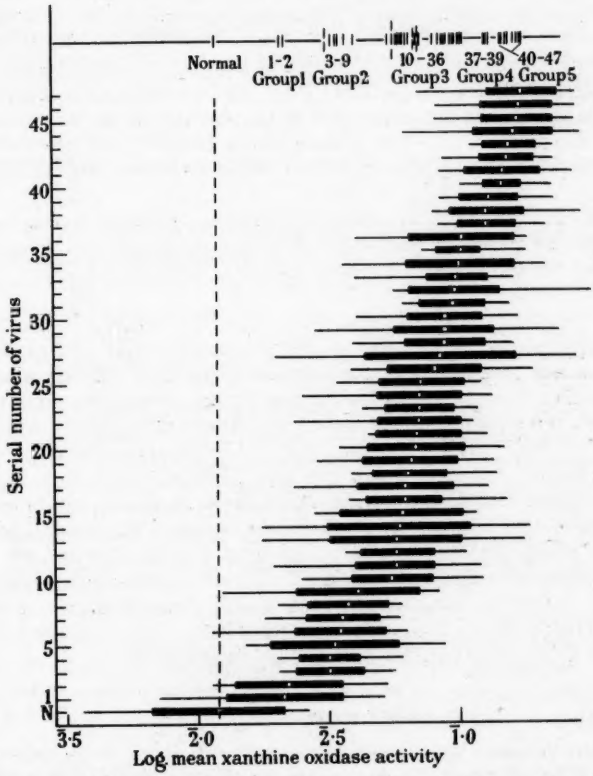

FIG. 1.—Mean, range and standard deviation of xanthine oxidase activity of normal mouse brain and of brains infected with neurotropic viruses. *Abscissa :* logarithm of xanthine oxidase activity ; narrow line, range ; broad line, extent of 1 standard deviation on either side of mean. *Ordinate :* normal brain (N), followed by serial numbers of viruses. *Upper line :* mean xanthine oxidase activities of normal and infected brains, showing occurrence in groups.

activity of the brain increases exponentially ; this can be seen in the growth curve of 17D xanthine oxidase activity reported earlier (Bauer, 1947*a*), and has been more fully established in studies to be reported separately ; the activity therefore rises very rapidly towards the end of the survival period and the geometric mean is the only one which can appropriately be taken in these circumstances. A logarithmic scale has been chosen for the abscissa in order to show the standard deviations, which were calculated from the logarithms of the optical densities.

From the Figure it will be seen that the mean xanthine oxidase activity is significantly increased for every virus investigated, and ranges from 1·6 to 13·4 times the normal value. Since the viruses included in this study comprise a considerable part of those actually known it is reasonable to infer that a rise of xanthine oxidase activity is a general feature of virus multiplication in mouse brain.

The Xanthine Oxidase Group Diagram

The Figure also shows that the extent of the rise of xanthine oxidase activity is not the same for all viruses, and that they can be arranged in order of increasing activity. This is brought out more clearly by plotting the mean xanthine oxidase values along the upper axis. The Figure shows further that the mean values for different viruses do not form a continuous or random series, but fall into the following groups :

Group 1 (serial numbers 1, 2) ; Senger, Bwamba ; range of means 0·0214–0·0219.

Group 2 (3–9) ; pseudolymphocytic choriomeningitis, neuro-vaccinia, encephalomyocarditis, Fantz, California, Bunyamwera, neurotropic Rift Valley fever ; range 0·0371–0·0406.

Group 3 (10–36) ; MM, Mengo, GD VII, TO, MEFI, Columbia-SK, PR8, NWS, Haemagogus A, Anopheles B, herpes, neurotropic yellow fever, Leucocelaenus, Sabethes, Anopheles A, JWS, rabies (Flury), JHM, Wyeomyia, Haemagogus B, Semliki Forest, neuro-Mel, neuro-Kunz, meningopneumonitis, sandfly fever (Naples), lymphogranuloma venereum, Ntaya ; range 0·0548–0·105.

Group 4 (37–39) ; Ilhéus, dengue type 2, Uganda S ; range 0·126–0·135.

Group 5 (40–47) ; sandfly fever (Sicilian), García, Colorado tick fever, neuro-Swine, yellow fever (17D), West Nile, dengue type 1, Zika ; range 0·149–0·177.

By means of the t test the differences between the means of the highest member of a group and the lowest member of the next group are found to be significant (normal-Senger, $p = 0{\cdot}009$; Bwamba-pseudolymphocytic choriomeningitis, $p = 0{\cdot}013$; neurotropic Rift Valley fever-MM, $p = 0{\cdot}036$; Ntaya-Ilhéus, $p = 0{\cdot}04$; Uganda S-sandfly fever (Sicilian), $p = << 0{\cdot}001$). The existence of the groups as discrete entities is therefore established.

Invariance of the Mean

Is the mean value a characteristic property of a given virus, or would a widely different mean be obtained if a second group of brains were subjected to assay ? This can be settled in two ways. Firstly, the mean was not obtained from a continuous series of assays, but from the combined values of two or more series or assays with the same virus separated by a considerable period of time. The separate means of the sub-series can then be calculated, and are found to be in good agreement.

Examples (1). *García* ; 11 determinations, mean 0·147 ; 10 determinations, mean 0·147 ; final mean, 28 determinations, 0·151.

(2). *Ilhéus ;* 12 determinations, mean 0·125 ; 6 determinations, mean 0·130 ; final mean, 28 determinations, 0·126.

Secondly, several examples are known of groups of viruses which have been isolated in different parts of the world at different times and which are known under different names, but which have subsequently been shown to be indistin-

guishable by serological cross-reactions. These viruses have been examined separately in the present investigation under their original names, yet still give the same xanthine oxidase value. Certain viruses which are closely related but not identical also give closely similar mean values.

Examples (serial number and mean in brackets):

(1) EMC group: (*a*) *encephalomyocarditis* (5; 0·0333); *Fantz* (6; 0·0345). (*b*) *MM* (10; 0·0482); *Mengo* (11; 0·0504).

(2) Mouse encephalomyelitis group: (*a*) *GD VII* (12; 0·0575); *Haemagogus A* (17; 0·0642). (*b*) *Leucocelaenus* (22; 0·0709); *Sabethes* (23, 0·0716). (*c*) *Wyeomyia* (28; 0·0892); *Haemagogus B* (29; 0·0903).

(3) Influenza group; (*a*) *PR8* (15; 0·0621); *NWS* (16; 0·0625). (*b*) *Neuro-Mel* (31; 0·0959); *Neuro-Kunz* (32; 0·0987).

(4) Psittacosis group; *Lymphogranuloma venereum* (35; 0·115); *meningopneumonitis* (36; 0·123).

By means of the t test it was found that the difference between the mean values in the sub-divisions of the above groups was not significant. The evidence therefore suggests that the mean value for a given virus is invariant and a fundamental characteristic of the virus.

Existence of Fine Structure within the Groups

The mean values in a group vary within a range which is quite considerable in group 3: this might occur in two ways; either all the viruses in a given group have the same mean, in which case the range would represent the extent of variation to be expected in replicate determinations, or else there is a real difference between the means of some or all viruses in a given group. Application of the t test to the differences between means enabled the following conclusions to be drawn.

Group 1.—The difference between the means of Bwamba (1) and Senger (2) is not significant.

Group 2.—The difference between the means of the extreme members of the group (pseudo-lymphocytic choriomeningitis, 4; neurotropic Rift Valley fever, 9) is not significant ($p = 0·35$). The group is therefore homogeneous.

Group 3.—Some evidence of inhomogeneity in this group has already appeared in the discussion of the invariance of the mean. The following sub-groups can be distinguished:

(i) Mengo, MM, Columbia-SK.
(ii, *a*) GD VII, Haemagogus A; Leucocelaenus, Sabethes;
(ii, *b*) Wyeomyia, Haemagogus B.

The difference in mean xanthine oxidase value between GD VII and Sabethes is not significant, and these viruses therefore form a distinct sub-group; the difference between the means of this sub-group and the Wyeomyia-Haemagogus B sub-group is significant ($p = 0·03$).

The influenza viruses fall into 2 distinct sub-groups:

(iii, *a*) PR8, NWS.
(iii, *b*) neuro-Kunz, neuro-Mel.

The difference between the means of the 2 sub-groups is significant ($p = 0·004$).

The difference between the means of the extreme members of group 3 (MM, 10; Ntaya, 36) is highly significant; this group is therefore not homogeneous,

but represents an aggregation of distinct means, apart from those which can be recognised as characteristic of the above sub-groups.

Group 4.—The difference between the means of the extreme members of the group (Ilhéus, 37 ; Uganda S, 39) is not significant ($p = 0{\cdot}278$) and the group is therefore homogeneous.

Group 5.—The difference between the extreme means (sandfly fever, Sicilian strain, 40 ; Zika, 48) is significant ($p = 0{\cdot}036$), and the group is therefore not homogeneous. The four highest members of the group (yellow fever, 17D ; West Nile ; dengue I, Hawaiian strain ; Zika ; 45–48) are antigenically related, and the difference between the means of the extreme members is not significant ($p = 0{\cdot}2$) ; these viruses therefore form a distinct sub-group.

Consideration of Certain Individual Cases

(*a*) *The neurotropic* (21) *and* 17*D* (44) *strains of yellow fever virus*, which can be distinguished by differences of behaviour in laboratory animals, fall into different xanthine oxidase groups (3 and 5 respectively).

(*b*) *Mouse encephalomyelitis viruses.* These viruses have hitherto been considered to be identical from the results of cross-neutralisation (Smithburn, 1952) and cross-fixation (Kerr, 1952) tests, but they can be classified into two distinct types on the basis of their mean xanthine oxidase value. A slight difference in behaviour has been reported for Haemagogus B, which differs from the other members of the group in that it is not antigenic in monkeys (Smithburn, 1952).

(*c*) *Sandfly fever viruses.*—The Naples (34) and Sicilian (40) strains of sandfly fever virus, which are antigenically unrelated, belong to different groups (groups 3 and 5 respectively).

(*d*) *Encephalomyocarditis group.*—These viruses fall into 3 separate groups. Senger (1) occurs alone in group 1 ; encephalomyocarditis (5) and Fantz (6) in group 2 ; MM (10), Mengo (11) and Columbia-SK (15) in group 3. They have hitherto been considered to be closely related or identical on the basis of cross haemagglutination-inhibition tests (Dick, 1949 ; Warren, Smadel and Russ, 1949 ; Sanders and Taverne, personal communication, 1955) but it is evident that these viruses can be separated into 3 types on the basis of their mean xanthine oxidase values. If the mean normal xanthine oxidase value is subtracted from the weighted means of the 3 types the following values are obtained : type 1, 0·00925 ; type 2, 0·02173 ; type 3, 0·04503. These values approximate fairly closely to a 2 : 2 : 4 ratio. While this may be fortuitous, it nevertheless suggests that there is some kind of quantisation underlying the production of the extra xanthine oxidase activity which appears during infection, based possibly on a phenomenon allied to polyploidy, or on a limited series of binary fissions at some state in the multiplication process.

(*e*) *Meningopneumonitis* (33) and *lymphogranuloma venereum* (35) have a characteristic mean xanthine oxidase value. They therefore resemble the true viruses in this respect, although there is some doubt as to their true systematic position.

(*f*) *Influenza viruses.*—These can be separated into 3 distinct types : (1) PR8, (16, 0·0621) ; NWS (17, 0·0625) : (2) Neuro-Mel (31, 0·0959) ; Neuro-Kunz (32, 0·0987) : (3) Neuro-Swine (43, 0·159). The above 3 recombinants show a xanthine oxidase mean which differs markedly from that of the parent NWS strain. The recombinant JWS (25, 0·0733) has a mean lying between those of the parents NWS and JHM (27, 0·0862), the ratio of the JWS-NWS difference to the JHM-NWS difference being 0·4565 : 1.

(*g*) *Dengue.*—The 2 strains of dengue virus, (type 1, 46 ; type 2, 38) which are immunologically distinct, occur in different xanthine oxidase groups (5 and 4 respectively).

The Origin of the Extra Xanthine Oxidase

The possibility that the increase of xanthine oxidase activity during a virus infection might be due to the ingress of inflammatory cells has been discussed in earlier work, and evidence has been produced to exclude this possibility. The fact that the mean is invariant and falls into 5 groups cannot be readily explained

by assuming a quantisation of the inflammatory response, and it is therefore likely that the appearance of the extra xanthine oxidase is related in a more intimate manner to the process of virus multiplication. The simplest explanation would be that the extra xanthine oxidase is situated in the virus particles. If this were the case, there should be some simple relationship between the mean enzyme activity and the amount of virus present. The latter quantity is proportional to the titre, and in Fig. 2 the logarithm of the titre of some of the viruses has been plotted against the logarithm of their mean xanthine oxidase activity; some titres have been taken from Smithburn (1952) and others have been specially determined for the purpose. It will be seen that in each group the titre ranges over about 4 log units with very little change in the mean xanthine oxidase activity, and the latter therefore cannot in general be proportional to the number of virus particles. If this were the case the points of the individual viruses would all lie on a line of unit slope.

The viruses on the right of the diagram do lie near such a line, however, and the possibility of a direct connection between enzyme activity and number of virus particles is worth exploring in these cases. From a consideration of the molecular weight of triphenyltetrazolium chloride, the molecular extinction coefficient of triphenylformazan, Avogadro's number, the turnover number of xanthine oxidase and the experimental conditions it can readily be shown that an activity represented by an ordinate of $\bar{2}{\cdot}9147$ in Fig. 2 corresponds to 10^{13} molecules of xanthine oxidase in the whole brain. The titre of a virus as determined by intracerebral inoculation of mice is less than the total number of particles in the inoculum by a factor of about 100, since not all particles remain at the site of inoculation and the probability of infection is less than unity; the virus content of the whole brain can be obtained by multiplying by a dilution factor of 160. The number of particles in the brain is therefore given by increasing the abscissae by 4·2 log units; an observed titre of 10^9 then represents $1{\cdot}6 \times 10^{13}$ virus particles. AB is drawn with unit slope through points corresponding to equal numbers of enzyme molecules and virus particles. For the viruses which lie near this line (Senger, encephalomyocarditis, Mengo, Semliki Forest, Ilhéus) one particle corresponds to one enzyme molecule, at least to within a factor of 10 or so. This cannot be the case for those viruses which lie to the left of AB, since the number of enzyme molecules then exceeds the number of virus particles to the same extent as the titre falls short of the titre read from AB. It is of interest that no viruses lie to the right of AB, where the number of virus particles would greatly exceed the number of enzyme molecules, and a maximum 1 : 1 correspondence between virus and enzyme may therefore be the explanation of the fact that virus titres based on an inoculum of 0·025 or 0·033 ml. never exceed 10^9 in mice. By analogy with bacteria it is natural to suppose that the enzyme molecules are contained in the virus particles, but the enzyme content of different viruses would then have to vary over a 10,000-fold range, and it is in any case uncertain whether viruses possess much intrinsic enzyme activity. Intracerebral injection of a large dose of PR8 virus produces a rise in xanthine oxidase activity to 5 times the normal level, although there is little or no formation of infective particles. The enzyme is therefore associated with an earlier stage of virus multiplication and cannot be present inside mature particles in this instance. Attempts to demonstrate the presence of xanthine oxidase in preparations of PR8 virus concentrated from allantoic fluid have so far been unsuccessful, and the weight of evidence

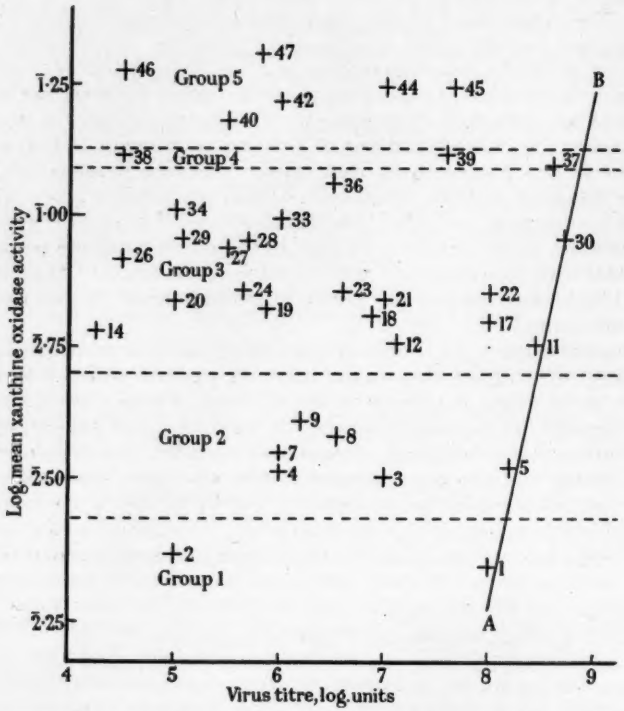

FIG. 2.—Relation of logarithm of mean xanthine oxidase activity of viruses to the infectivity titre.

therefore suggests that xanthine oxidase is not present in virus particles but is associated with their formation.

If the mean xanthine oxidase activities are plotted against the logarithm of the product of the titre and the volume of the virus particle the distribution of points obtained is essentially similar to that of Fig. 2. The amount of enzyme is therefore not proportional to the amount of virus material present. The evidence therefore suggests that the enzyme is closely associated with the virus but is not in it ; it could possibly be associated with matrix, soluble antigen, host component, or incomplete virus, and it is worth noting that soluble antigens are prepared in such a way that they would undoubtedly contain xanthine oxidase, although the enzyme would gradually become inactivated during processing, and that their titres vary over a restricted range of the same order of magnitude as that of the xanthine oxidase mean. This varies over a 10-fold range, whereas virus titres vary over a range of 10,000-fold, and it may be postulated that the enzyme is associated with potential virus material which matures to the stage of infectivity to widely differing extents, with 1 enzyme molecule corresponding to 1 virus particle when the development of infectivity reaches its maximum.

The Relation of the Xanthine Oxidase Groups to Other Classifiable Functions of Viruses

Antigenic relations.—Although the groups all include viruses which are antigenically related, and they separate viruses considered to be identical, they nevertheless show a considerable correlation with antigenic relationship in the widest sense. Thus, lymphogranuloma venereum (35) and meningopneumonitis (33) both occur in group 3 ; NWS (17), PR8 (16), neuro-Kunz (32), neuro-Mel (31) and JWS (25) occur in group 3, and certain viruses which show weak unilateral cross-reactions (Zika, 47 ; West Nile, 45 ; Dengue I, 46) fall in group 5. The two strains of yellow fever, however, are widely separated (neurotropic (21), group 3 ; 17D (44), group 5), and one strain of influenza (neuro-Swine (43), group 5) does not fall into group 3 with the others. The case of the EMC group of viruses has already been discussed.

Haemagglutination groups.—Haemagglutination can easily be demonstrated with viruses of the influenza, encephalomyocarditis, vaccinia and mouse encephalomyelitis groups ; Casals and Brown (1954) have demonstrated two types of haemagglutinin in association with certain other viruses, and Clarke and Theiler (1955) have found a haemagglutinin associated with Bunyamwera virus. These haemagglutinins fall into 7 groups, according to the conditions necessary for their action, and their relation to the xanthine oxidase groups is shown in Table I. Although there is no absolute correlation, there is some association between

TABLE I.—*Relation between Xanthine Oxidase Groups and Type of Haemagglutination*

Type of haemagglutination.	Xanthine oxidase group.	Conditions for haemagglutination		
		Cells.	pH.	Temperature.
Casals and Brown type B				
Dengue I, West Nile, Yellow fever	5	1-day chick	7·0	4°, 22°
Dengue II, Ilhéus, Uganda S	4	„ „	7·0	4°, 22°
Ntaya	3	„ „	7·0	4°, 22°
Casals and Brown type A				
Semliki Forest	3	„ „	6·4	37°
Influenza				
Neuro-swine	5	Chick	—	—
PR8, NWS, Neuro-Kunz, Neuro-Mel	3	„	—	—
Mouse encephalomyelitis				
GD VII, Haemagogus A, Haemagogus B, Leucocelaenus, Sabethes, Wyeomyia	3	Human group O	—	4°
Encephalomyocarditis				
Mengo, MM, Columbia-SK	3	Sheep	—	0°–37°
Fantz, encephalomyocarditis	2	„	—	0°–37°
Senger	1	„	—	0°–37°
Clarke and Theiler				
Bunyamwera	2	Chick	6·0	4°–37°
Vaccinia				
Neurovaccinia	2	„	—	37°

xanthine oxidase group 3 and haemagglutination of the influenza, encephalomyocarditis and mouse encephalomyelitis type, whereas viruses of Casals and Brown's type B, and neurovaccinia and Bunyamwera, fall in the higher and lower xanthine oxidase groups respectively.

There is of course no basis for supposing that there is any direct connection between xanthine oxidase activity and occurrence of haemagglutination; the members of each haemagglutination group show extensive cross-reactivity, and both types of group phenomenon may only be an expression of a basic serological affinity. If this is the case, the xanthine oxidase group phenomenon reveals a wider relationship, and further work might be expected to demonstrate haemagglutinins in other group 4 and 5 viruses (sandfly fever, Sicilian strain, 40; García, 41; Colorado tick fever, 42; and Zika, 47).

Receptor gradient.—Five neurotropic strains of influenza virus have been examined, together with PR8 virus assayed at the height of the neurotoxic effect. Five strains fall into group 3 (PR8, 16; NWS, 17; JWS, 25; neuro-Mel, 31; neuro-Kunz, 32) and 1 into group 5 (neuro-Swine, 43). As far as data are available these viruses occur in the receptor gradient in the following order (Burnet, personal communication, 1955; Burnet and Edney, 1951), NWS < PR8 < neuro-Mel < neuro-Swine. When arranged according to increasing mean xanthine oxidase value the following order is obtained: (NWS = PR8) < JWS < (neuro-Mel = neuro-Kunz) < neuro-Swine. The viruses which are common to both sequences therefore occur in the same order. It is not possible to draw any definite conclusions from such a limited number of examples but there is at least the possibility of a relation between xanthine oxidase mean order and receptor gradient position, and it might conceivably be that the receptor gradient, which has not yet been satisfactorily explained, is a particular case of a more general and possibly fundamental phenomenon.

TABLE II.—*Relation between Xanthine Oxidase Groups and Oncolysis*

Xanthine oxidase group.	Virus.	Tumour. KB.	HeLa.	Sarcoma 180.	Ehrlich carcinoma.	Methylcholanthrene tumour.	E0771 adenocarcinoma.
5	West Nile	..	+	+	±	±	+
4	Ilhéus	..	..	+	±	±	±
3	Semliki Forest	..	..	0	0	0	0
	Rabies	±	..	..	..	..	..
	Yellow fever	±	..	..	..	..	..
	Herpes	+	+	..	..	..	..
	Poliomyelitis	+	..	..	..	..	..
2	Bunyamwera	..	..	0	+	0	0
	Encephalomyocarditis	+	..	..	..	..	..
	Pseudo-LCM	..	+	..	..	..	..
	Vaccinia	+	+	±	..	..	..

Oncolysis or cytopathogenic effect: + = present; ± = slight or delayed; 0 = absent.

Oncolysis.—The type of interference between viruses and tumours which is responsible for oncolysis and cytopathogenic effects could also be used as a means of grouping viruses. Published instances of oncolysis have been reviewed by

Siegert (1955), and Table II has been constructed from his data and from the work of Eagle, Habel, Rowe and Huebner (1956) and Scherer and Syverton (1954, 1955). When the viruses are arranged in xanthine oxidase mean order there is a tendency for the oncolytic or cytopathogenic property to be associated with a particular group; thus the group 2 and group 3 viruses are cytopathogenic to KB and HeLa cells, whereas the oncolytic effect against the other tumours is mainly confined to viruses of groups 4 and 5, although further work with other tumours and viruses would be necessary to establish whether there is any general relation between xanthine oxidase groups and oncolysis. It is customary to refer to the "tumour spectrum" of the oncolytic property of a virus, and it might also be possible to establish a virus spectrum for any particular tumour, and from a consideration of the xanthine oxidase mean value of the viruses to arrange tumours in an order; in this connection it would be of interest to determine the xanthine oxidase activity of the tumours concerned, in order to find whether they have a characteristic mean value which bears some relation to the xanthine oxidase groups of the viruses.

Function of the Xanthine Oxidase

Nothing has been said so far about the function of the xanthine oxidase which appears during virus multiplication. The enzyme is usually considered to be responsible for the catabolism of purines which arise as breakdown products of nucleic acids, and this is presumably its main function in such organs as the liver and intestine. The constant appearance of the enzyme during virus multiplication in an amount characteristic of the particular virus concerned suggests that xanthine oxidase is not an unimportant by-product but is in fact essential to the process of virus synthesis. It seems improbable that the oxidation of hypoxanthine to uric acid could be important in virus multiplication, particularly as the biosynthesis of purine nucleotides is known to go along other paths, and it is more likely that one of the other functions of the enzyme, such as the oxidation of aldehydes or of reduced co-enzyme I, is the reaction for which it is required; acetaldehyde, for instance, could be oxidised to acetate and then be built up into fatty acids, enter the citric acid cycle, or undergo other important metabolic transformations. The connection between xanthine oxidase activity and iodination reactions and thyroxine formation may also be of importance in virus multiplication.

Other evidence also suggests that xanthine oxidase may be of greater importance than is generally realised. For instance, xanthine oxidase is present in all tissues in mice, often in high concentration, and an enzyme which parallels the cytochrome oxidase system in the extent of its distribution is likely to be of equal importance. The molecule of xanthine oxidase contains 1 atom of molybdenum, 2 molecules of flavine adenine dinucleotide and 8 atoms of iron (Richert and Westerfeld, 1954). The activity of metal ions of various kinds is of great importance in the functioning of biological systems, and probably represents the final stage in the evolution of a type of reaction which arose when life was first evolving from organic substances and metal ions in solution in the hydrosphere. The importance of a particular element in life processes can be gauged from its relative abundance; an element which is abundant in nature may or may not be of great biological importance, but an element which is rare and which is nevertheless of widespread occurrence in biological systems is probably subserving a function of fundamental importance. The relative cosmic abundances of the biological elements are shown in Table III,

TABLE III.—*Relative Abundances of the Biological Elements*

H, $3{\cdot}5 \times 10^6$
O, 220,000; N, 160,000
C, 80,000
Mg, 8,870; Fe, 7,250; S, 1800
Ca, 660; Na, 462; Cl, 170
P, 90; Mn, 75; K, 69; Co, 22
Cu, 7·1; Zn, 2·6; V, 2·4
Mo, 0·072

which has been constructed from data of Urey (1952). The figures represent the relative abundances of the elements in the universe derived from studies of the composition of meteorites, the earth, and stars of various types, and are based on an arbitrary figure of 10,000 for silicon. The horizontal lines in the Table represent successive powers of 10. Molybdenum is seen to be by far the least abundant of the biological elements, being at least 300 times rarer than zinc and vanadium, and its preferential incorporation into biological systems which have survived until the present day is difficult to explain unless it fulfils some function of much greater significance than the mere preparation of unwanted purines for disposal. From the closeness of its association with the multiplication of virus it is tentatively suggested that xanthine oxidase may be required for the formation of new nucleoprotein.

Practical Applications of the Xanthine Oxidase Group Phenomenon

Application to systematics

Since all the viruses which have been examined fall into 5 groups, the possibility arises that the xanthine oxidase group phenomenon might be of use in the classification of viruses. A classification erected on this basis would be a reflection of a single natural property and would therefore lack the artificiality inseparable from the application of Linnean and non-Linnean binomials to viruses; it would be comprehensive from the outset, since an infinity of xanthine oxidase mean values remains to be occupied by viruses yet to be discovered, even if new groups arise, or values which lie between the existing ones. A further advantage of such a system would be the fact that it conveniently associates viruses which have so far defied association, such as pseudo-lymphocytic choriomeningitis, neurovaccinia, neurotropic Rift Valley fever, encephalomyocarditis, Bunyamwera and California, which fall close together in group 2. It nevertheless associates viruses which are associable by conventional means, such as the haemagglutinating viruses of Casals which fall into groups 4 and 5. A disadvantage of such a classification, however, is the fact that the xanthine oxidase groups separate viruses which are obviously similar, such as the encephalomyocarditis viruses which are distributed between groups 1, 2 and 3, the neurotropic and 17D strains of yellow fever virus, and different strains of influenza virus. The classification would moreover be insufficiently detailed, in that some further subdivision would be required for the specification of antigenically unrelated viruses which fall into the same group; it would therefore correspond at the most to a separation into the equivalent of genera. Virology has advanced a long way in the absence of any classification other than empirical ones based on laboratory procedures, and it is doubtful whether there is any real need for one at all, but a classification based on the xanthine oxidase groups would at least have some theoretical justification.

Use in typing

Examples have already been given to show how the determination of the xanthine oxidase mean can distinguish between viruses which cannot be separated by conventional methods, and this fact could be used as the basis of a typing procedure. The encephalomyocarditis viruses, for example, can be separated into 3 types and the mouse encephalomyelitis viruses into 2 types ; examination of a sufficiently large number of wild strains of virus might disclose other examples of the same phenomenon, and typing based on this method might be of some use in epidemiological work.

Detection and isolation of new viruses

The rise of xanthine oxidase activity in the brains of mice exhibiting the neurotoxic effect of intracerebral inoculation with PR8 virus shows that the formation of infective virus in any quantity is not an essential condition for the rise in enzyme activity. A rise can moreover be detected during the early days of infection with other viruses some time before the titre of infective virus approaches its maximum value. These findings suggest that a determination of xanthine oxidase activity might have some application in the detection and titration of viruses which are not pathogenic for the usual laboratory animals. For example, infective hepatitis virus might undergo a few cycles of multiplication in mouse brain without producing any obvious signs of its presence, and if this were so it should be possible to detect an increase in xanthine oxidase activity. Coryza virus present in bacteriologically sterile nasal secretions might be similarly detected after intracerebral inoculation in mice, or after the inoculation of chick embryos by various routes. Reference to Fig. 2 will show that very high xanthine oxidase values can occur even although the virus has only multiplied to a low titre, and the presence of a virus might be detectable in the absence of any extensive multiplication.

SUMMARY

In infections of mouse brain with 47 neurotropic viruses the xanthine oxidase activity is increased in all cases, the extent of the increase ranging up to 13 times the xanthine oxidase activity of normal mouse brain.

The geometric mean of the xanthine oxidase activity of the brain at the height of infection is invariant and characteristic of the particular virus.

When arranged according to the order of the xanthine oxidase mean value the viruses fall into 5 separate groups.

There is some evidence that the xanthine oxidase groups can be correlated with other functions of viruses, such as antigenic relationship, haemagglutination, the receptor gradient and oncolysis.

In certain instances the number of enzyme molecules which appear in the brain is approximately equal to the number of virus particles. The formation of new enzyme is closely associated with the process of virus multiplication. The enzyme cannot in general be situated inside the virus particle, but may be related to soluble antigen.

The xanthine oxidase group phenomenon can be used as a means of distinguishing between certain viruses hitherto considered to be identical ; it is suggested that it might be of some value in typing, and in the isolation of viruses which are not apparently pathogenic for laboratory animals.

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Addendum.—After this work had been finished we were informed by Dr. F. O. MacCallum that the Sandom strain of pseudo-lymphocytic choriomeningitis virus is a neurotropic variant of ectromelia. It is therefore of particular interest to note the close agreement of the mean (0·0316) with that of neurovaccinia (0·0318).

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PRESSURE FLUCTUATIONS IN THE CEREBROSPINAL AXIS OF RABBITS AS A POSSIBLE PHYSIOLOGICAL MECHANISM FOR VIRUS DISPERSAL IN THE CENTRAL NERVOUS SYSTEM

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IT is now recognised that many neurotropic viruses spread rapidly through the central nervous system from their site of entry in the brain or spinal cord, although little is known about the means by which this dispersal is brought about. The present study records some observations on variations in pressure within the cranial cavity and vertebral canal as a result of respiratory and cardiac activity which might contribute to the spread of such agents. It is suggested below that the asynchronous changes in pressure due to differences in the instant of impact of the arterial pulse at different levels of the cerebrospinal axis may result in frequent small displacements of its interstitial constituents whose cumulative effect over a period of many hours may bring about considerable translocation of any contained virus, toxin or foreign protein.

MATERIALS AND METHODS

Animals.—Rabbits of various strains weighing 2·2–3·5 kg. were used.

Operative procedures.—All operations were carried out under general anaesthesia produced by intravenously administered veterinary Nembutal (0·4 ml./kg.) supplemented by open ether. Pulse recordings from the posterior cranial fossa were obtained by introducing a No. II serum needle 40 mm. long obliquely through the atlanto-occipital membrane in the midline. Similarly, pulse recordings were made from the thoracic and lumbar regions of the spinal cord by inserting the needle into the vertebral canal between the neural arches of the 2nd and 3rd thoracic and the 6th and 7th lumbar vertebrae respectively. Access at these levels was obtained through short mid-line skin incisions: the erector spinae muscles were separated from the vertebral spines and retracted laterally. A radiograph of the carcass of an animal with needles inserted at the three sites used showed that the point of the needle in the posterior cranial fossa was 8·5 cm. from that in the thoracic region, and that this in turn was 26 cm. from the point of the needle in the lumbar region.

All pulse recordings were made with rabbits in the prone position.

Pulse recordings.—Recordings of pressure changes within the central nervous system were made with a Southern Instrument Company blood pressure recording apparatus Type RB16, with a 50 mm. Hg capacitance manometer head. This head was connected to the No. II serum needle by a short length of polythene tubing (0·75 mm. internal and 1·5 mm. external diameters), the whole system being filled with saline. The instrument was used on its maximum sensitivity range (0–12·5 mm. Hg) and its output was fed to one beam of a Cossor 1049 double beam oscillograph fitted with a long-persistence cathode ray tube. When pulses in the carotid artery were recorded, a Technitrol Lilly 115–1 capacitance manometer fitted with a 400 mm. Hg pressure head was used: its output was similarly fed to the oscillograph.

Electrocardiograms.—For the electrocardiographic records, electrode clips were attached to the edges of the interscapular and lumbar skin incisions. The records were made with an amplifier of the type described by Boyd and Eadie (1952), the output of which was passed to the other beam of the oscillograph.

Recording methods.—Photographic records were made with a continuous film camera from a second cathode ray tube in parallel with the oscillograph. Time marking, at 20 m.sec. intervals, was provided by a small cathode ray tube mounted immediately above the main camera tube. Records were made on Ilford 5B52 film driven at 75 mm./sec. Measurements on the film records were made with a cursor running on a scale graduated in mm. The time intervals between the onset of ventricular systole and the onset of the "arterial" pulse at each of the three levels in the cerebrospinal axis were determined by measuring the distance from the start of the R wave in the electrocardiogram to the start of the "arterial" pulse wave in the central nervous system tracings. Twenty such measurements were made from each of the records and the average distance determined. From this and from the speed of the film, the time interval was calculated.

RESULTS

Pulsations in the posterior cranial fossa and the carotid artery.

Pressure records obtained from the posterior cranial fossa in the immediate vicinity of the medulla oblongata usually present two major oscillations during the period of a single cardiac cycle. Of these, one component is conspicuous for its amplitude and the steepness of its rise; for these reasons, it was regarded as representing the arrival of the main arterial pulse wave within the cranial cavity. To test this inference, pressure records were made simultaneously from the posterior cranial fossa and from the internal carotid artery. Tracings thus obtained are shown in Fig. 1. From this, it can be seen that the rise in pressure

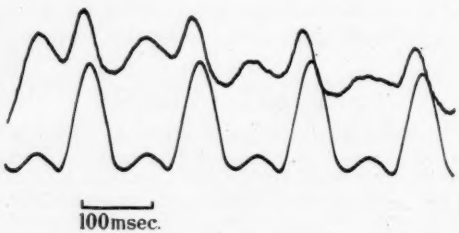

FIG. 1.—Simultaneous pressure recordings from the posterior cranial fossa (upper trace) and the internal carotid artery (lower trace).

takes place at almost the same instant at both sites, although if the carotid cannula was directed towards the heart the arterial pulse slightly preceded the intracranial one, whereas if it was directed towards the circle of Willis, the order of the two pulses was reversed. Such differences are to be anticipated if with the former arrangement the arterial pulse reached the cannula directly and with the latter it had to retrace its course backwards from the large arterial anastomoses within the skull.

Pulsations in the posterior cranial fossa and in the vertebral canal in the thoracic and lumbar regions

Pressure waves due to the arterial pulse can be recorded from the vertebral canal as well as from the posterior cranial fossa, although their amplitudes are less and their instants of arrival at the three levels are different. To determine

the delay between the onset of ventricular systole and the rise of the " arterial " pulse at the three sites, each pressure tracing was paired with a simultaneous electrocardiogram. Three such tracings are shown in Fig. 2, and the findings from six rabbits are given in the Table.

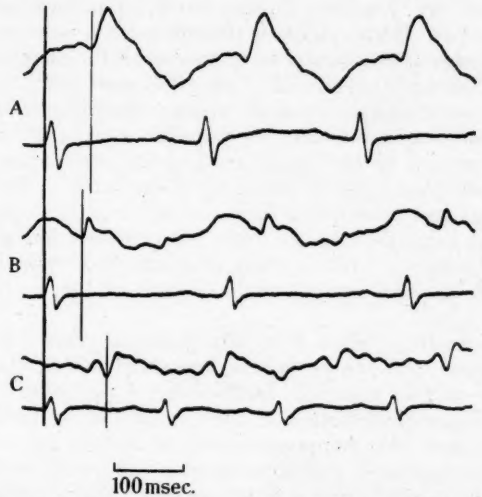

FIG. 2.—Pressure recordings obtained from : A, the posterior cranial fossa ; B, the vertebral canal at the 2nd–3rd thoracic interspace ; and C, the vertebral canal at the 6th–7th lumbar interspace. Each pressure trace is paired with a simultaneous electrocardiogram. The first vertical line indicates the onset of ventricular systole as shown by the electrocardiogram. The second vertical line in each of the three pressure traces indicates the onset of the " arterial " pulse wave at that site.

TABLE.—*The Intervals (in milliseconds) between the Onset of Ventricular Systole and the Arrival of the "Arterial" Pulse at Three Levels of the Cerebrospinal Axis.*

	Posterior cranial fossa.	2nd to 3rd thoracic interspace.	6th to 7th lumbar interspace.
	53	—	87
	59	—	91
	65	47	97
	62	52	95
	68	41	85
	53	49	92
means	60	47	91

From this Table, it can be seen that the arterial pulse first impinges on the thoracic segments—a finding probably explicable from their close proximity to the aortic arch—next (nearly 15 m. sec. later) in the posterior cranial fossa, and lastly (about 45 m. sec. later) on the lumbar segments. It seems worthy of note that these time intervals appear to be directly related to the distances along the cerebrospinal axis separating the three sites of pressure recording.

DISCUSSION

It has been the experience of many investigators that once a neurotropic virus has been injected at one site in the central nervous system, it spreads quickly to all its other parts. This phenomenon, termed " septinévrite ", was described in detail by Nicolau, Dimanesco-Nicolau and Galloway (1929) for the viruses of herpes, Borna disease, neurovaccinia, poliomyelitis and rabies. With all five viruses, an inoculation into one cerebral hemisphere of a monkey or rabbit led to its dissemination not only into the brain stem and spinal cord, but even into such distant nerve trunks as the sciatic. Not only do these viruses spread from the brain downwards, but they are capable of passing equally readily from the lumbar enlargement of the spinal cord to the brain stem. The problem of " septinévrite " has been fully reviewed by Doerr (1939). From the occurrence of both centrifugal and centripetal dispersal of virus in the cerebrospinal axis, therefore, it seems impossible to attribute the translocation of virus particles to any directional streaming ; rather their dissemination appears to be a random mixing dependent on some oscillatory movement of the medium in which they are contained.

Manometric recordings taken from the posterior cranial fossa and from the vertebral canal show that the structures contained within these relatively rigid cavities are subjected to constant oscillations of pressure. While a complete study of these pressure variations and their causes would involve much detailed analysis, it seems that two components can be readily identified : the first, of slow rise and low frequency, due to respiratory movements, and the second, a group related to the cardiac cycle in which there is a sharply rising wave due to the impact of the arterial pulse. It is this latter wave that is of chief interest in the present study since it has been found that the " arterial " pulsations recorded are not synchronous throughout the cerebrospinal axis, those in the posterior cranial fossa and in the lumbar region being notably later than that in the thoracic region.

The effects of phasic localised elevations in pressure in the contents of the vertebral canal must necessarily entail corresponding movements of any interstitial fluid that may be contained in the substance of the cerebrospinal axis. Although the extent of the movement imposed by a single arterial pulse must be small, its repetition in a rabbit half a million times daily might well promote the distribution of virus particles and toxins over major portions, if not the whole length, of the cerebrospinal axis, and thus provide a physiological mechanism both for the phenomenon of " septinévrite " with viruses and for the ascent of the spinal cord by tetanus toxin and other proteins (Wright, Morgan and Payling Wright, 1951).

SUMMARY

Pressure recordings in rabbits have been made from the posterior cranial fossa and from the vertebral canal in the upper thoracic and lower lumbar regions.

The arterial component of the observed pressure fluctuations has been identified at each site, and it has been found that the local rise in pressure due to the impact of the arterial pulse occurs at different instants at these three levels. The pulse wave arrives first in the thoracic region, later in the posterior cranial fossa and last in the lumbar region.

It is suggested that these constantly repeated and asynchronous fluctuations of pressure throughout the cerebrospinal axis may be a factor in the dispersal through the extracellular compartment of the central nervous system of such materials as virus particles and bacterial toxins from the site at which they were initially introduced.

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GASTRO-INTESTINAL MOTILITY AND ABSORPTION IN EXPERIMENTAL LIVER FAILURE

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RADIOLOGICAL studies on rats with acute hepatic insufficiency have revealed delayed gastric emptying and delayed passage of barium through the small intestine (Reynell, 1954). These findings may have some relevance to the practical problem of feeding patients with liver failure since the intragastric administration of 20 per cent glucose is often used in the treatment of hepatic coma (Sherlock, 1955). The introduction of a technique which measures simultaneously absorption and transit in the gastro-intestinal tract of the rat (Reynell and Spray, 1956) makes possible quantitative studies of gastro-intestinal function after the injection of glucose into the stomachs of rats with experimental liver failure.

METHODS

Adult male rats of the Wistar strain were used. Hepatic necrosis was produced in 18 animals by injecting 0·03 ml. of carbon tetrachloride into a branch of the mesenteric vein under ether anaesthesia (Reynell, 1953) and 11 control animals were subjected to an identical surgical procedure except that no carbon tetrachloride was given.

Gastric emptying, intestinal transit and intestinal absorption of glucose and iodide were measured simultaneously 24 hr. after operation by the method of Reynell and Spray (1956). All animals were intubated with a gum elastic catheter and 4 ml. of a solution containing approximately 3 mg. phenol red, 800 mg. glucose and 2 μc.^{131}I (as sodium iodide) was introduced into the stomach. A normal rat was killed immediately after intubation and used for control estimations of phenol red, glucose and iodide and 12 rats with hepatic necrosis and 5 sham-operated controls were killed 2 hr. after intubation. The stomach and small intestine were divided into the following four segments: (1) stomach, (2) proximal half of small intestine, (3) third quarter of small intestine, (4) distal quarter of small intestine. Each segment was homogenised and analysed separately for phenol red, glucose and iodide. Isotonic glucose in phenol red in doses of 4 ml. was given to 6 animals with hepatic necrosis and 6 controls. These rats were killed 1 hr. after intubation and gastric emptying and intestinal transit were measured.

Gastric emptying and intestinal transit were calculated as previously described (Reynell and Spray, 1956).

The *gastric emptying* is the percentage of administered phenol red which has left the stomach during the time since intubation.

The *intestinal transit* through a segment of small intestine is that percentage of the amount of phenol red entering the segment during the time since intubation, which has moved on to the next segment during the same period of time. It is thus a measure of the propulsive motility of that segment.

The *estimated intestinal absorption* of glucose or iodide is found by calculating the amount of test substance which has left the stomach during the time since intubation and subtracting the amount recovered from the small intestine at the end of this time. It is calculated from the following formula:

$$Ty\left(\frac{Py - Pa}{Py}\right) - Ti$$

Where Pa = amount of phenol red recovered from stomach of test rat, Py = amount of phenol red recovered from control rat killed immediately after intubation, Ti = amount

of test substance recovered from small intestine of test rat, Ty = amount of test substance recovered from animal killed immediately after intubation. (This formula slightly overestimates intestinal absorption as it assumes that the ratio of test substance to phenol red in the stomach remains constant, whereas in fact it tends to fall slightly owing to gastric absorption (Reynell and Spray, 1956)).

The *percentage intestinal absorption* of glucose or iodide is that percentage of the amount of test substance entering the small intestine during the time since intubation which has been absorbed by the time the animal is killed.

The *prothrombin index* was used as a measure of liver function and was determined by a one-stage micromethod using blood from a tail vein (Reynell, 1953). The index was obtained by dividing the time taken for the blood of a test animal to clot by the time taken for the blood of a normal animal to clot under the same conditions.

RESULTS

Animals given 20 per cent glucose (Table I)

Gastric emptying was delayed in all animals with liver damage and in 5 it was 20 per cent or less in 2 hr. as compared with an average of 63 per cent in the controls. The delay in gastric emptying bore some relation to the degree of liver damage as judged by the prothrombin index.

Intestinal transit was slow in all animals except in one which had so little liver damage that the prothrombin index was normal. In the most severely affected rats, the intestinal transit was greatly delayed.

Absorption of glucose and iodide.—The amounts of glucose and iodide absorbed by the small intestines of rats with liver damage during the 2 hr. experimental period were reduced in all animals, but this was due to the slow gastric emptying. The percentage of available glucose and iodide absorbed was not significantly reduced except in 3 of the most severely affected animals, 2 of which were clinically moribund when the measurements were made. The amount of glucose recovered from the distal half of the small intestine was always very small (less than 10 mg.) in the animals with liver damage and in the controls, and it is unlikely that any glucose reached the caecum. The small intestine was therefore capable of absorbing the whole of the glucose load.

Animals given isotonic glucose (Table II).

It could be argued that the delayed gastric emptying and intestinal transit observed in rats given 20 per cent glucose might be due to impaired ability to dilute the hypertonic solution with gastric secretions leading to a reflex inhibition of gastric emptying or delayed motility due to reduced distension. The experiments with isotonic glucose were designed to exclude this possibility, and the results show that both gastric emptying and intestinal transit are delayed under these conditions.

DISCUSSION

Our experiments suggest that as the liver fails, failure of the motor functions of the gastro-intestinal tract precedes failure of its absorptive capacity. Once nutritive material enters the small intestine it is absorbed normally unless the animal is so ill as to be almost moribund. The slow rate of absorption observed in these animals is due to delayed gastric emptying. If the same is true for man, it should be possible to nourish a patient with liver failure by mouth or gastric tube unless he vomits or develops progressive gastric distension. On the other

TABLE I.—*Gastric Emptying, Intestinal Transit and Intestinal Absorption of Glucose and Iodide in Rats with Liver Failure and Controls*

Rat number.	Pro-thrombin index.	Gastric emptying per cent.	Intestinal transit.			Glucose.		Iodide.	
			1st half.	3rd quarter.	4th quarter.	Estimated intestinal absorption mg./2 hr.	Per cent absorption.	Estimated intestinal absorption per cent dose given/ 2 hr.	Per cent absorption.
5 controls	1·0	63±12·1†	93±2·2	85·8±3·3	15±13	571±53·1	97±1·9	60±10·6	90·2±4·8
1	0·9	49	93	84	0	386	93	48	91
2	1·5	39	56	15	0	308	94	37	88
3	—	29	68	52	0	386	96	32	83
4	—	31	47	0	—	340	94	32	91
5	1·8	27	90	19	0	259	90	14	72
6	2·7	38	25	0	—	350	94	39	90
7	3·0	29	81	5	0	318	87	35	89
8	3·0	20	47	39	0	156	96	21	95
9	—	11	4	0	—	120	83	14	84
10	—	18	16	0	—	109	59	14	63
11*	3·0	15	16	0	—	53	49	8	56
12*	3·0	11	0	—	—	20	19	4	35

* animal moribund. — = no observation made. † S.E. of mean.

TABLE II.—*Gastric Emptying and Intestinal Transit after Administration of Isotonic Glucose.*

	Number.	Gastric emptying (per cent).	Intestinal transit.		
			1st half.	3rd quarter.	4th quarter.
Controls	6	73·3±2·3*	83·2±2·2	54·7±6·8	0
Liver failure	6	31·8±6·5	5·8±5·7	0	0

* S.E. of mean.

hand, the time relations of the action of drugs given by mouth or stomach tube to patients with severe liver damage may be altered. The action of a drug may be delayed and prolonged and this effect will summate with any prolongation of action due to failure of the detoxifying action of the liver.

The cause of the disturbed motor activity of the bowel is uncertain. Evidence that the autonomic nervous system is not directly involved has been presented (Reynell, 1954). It may be a manifestation of a non-specific response of the smooth muscle of the gastro-intestinal tract to injury or toxaemia, for there is experimental evidence that other toxic processes which may be accompanied by nausea and anorexia may have the same effect. X-irradiation can cause delayed gastric emptying (Goodman, Lewis and Schuck, 1952 ; Swift, Taketa and Bond, 1955) and Conard (1951) found delayed intestinal transit although Goodman *et al.* (1952) found no significant effect on the small intestine. Slow gastric emptying has also been observed after traumatic and histamine shock (Cordier and Pérès, 1950), crushing injuries (Whitely and Green, 1952) and toxic chemicals (Dieke, 1951), although intestinal transit has not been measured under these conditions. It has yet to be proved that these findings apply to species other than the rat, but there may be several pathological conditions in which the motor activity of the bowel may fail while its absorptive capacity remains relatively intact.

SUMMARY

Gastric emptying, intestinal transit and intestinal absorption of glucose and iodide have been studied in rats with acute liver failure produced by the injection of a small quantity of carbon tetrachloride into the mesenteric vein.

There was delay in gastric emptying and intestinal transit which was approximately proportional to the degree of liver damage as measured by the prothrombin index.

The rates of absorption of glucose and iodide were slow, but this was due to the slow gastric emptying. The absorptive capacity of the small intestine was not significantly impaired except in moribund animals.

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ANAPHYLACTIC SHOCK IN MICE

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ANAPHYLACTIC shock in the mouse was for many years a controversial topic until it was shown conclusively by Burdon (1937) that mice can be sensitised and shocked; this was later confirmed by Weiser, Golub and Hamre (1941), Mayer and Brousseau (1946), Nelson, Fox and Freeman (1950), and Wheeler, Brandon and Petrenco (1950). The reaction was not so dramatic as that seen in the guinea-pig, not all mice were shocked, and large doses of protein were used — 1·0 to 4 ml. of horse serum or egg-white, or 120 mg. of egg albumin for sensitisation; 0·5 to 2 ml. or 100 mg. for challenge; these features probably led to the phenomenon being regarded as something of a curiosity.

Much smaller sensitising and challenging doses—0·06 ml. and 0·15 to 0·05 ml. respectively of horse, guinea-pig, or rabbit serum—were found by McMaster and Kruse (1949) to be equally effective but again only in a proportion of mice. However, on examining the ears and feet of seemingly unshocked mice microscopically the authors noted vaso-spasm and arrest of circulation which they considered characteristic of anaphylactic shock. More recently, alum-precipitation was found by Solotorovsky and Winsten (1953) to enhance the activity of bovine albumin to such a degree that as little as 1 mg. and 0·016 mg. were sufficient respectively for sensitisation and shock.

The aim of the present paper is to show that at least one strain of mice, the W-Swiss, can be regularly sensitised to foreign protein, that the sensitising and challenging doses may be small, and that the antigen and the conditions of the experiment can be so chosen that the shock is invariably fatal. Other points investigated were the relative sensitising abilities of various proteins, the efficacy of various routes for sensitising and challenging, the effect of varying the number of sensitising doses, and the duration of sensitisation; also examined was the ability of an anti-histamine to prevent shock.

MATERIALS AND METHODS

Mice.—W-Swiss mice of both sexes, weighing 30–40 g., were used in all experiments: they were bred in the Department from stock obtained from the Agricultural Research Council's Field Station at Compton.

Bovine serum.—Whole blood from freshly killed animals was allowed to clot; the serum, after separation, was heated at 56° for 30 min. on 3 successive days and stored at − 20°.

Horse serum.—Burroughs, Wellcome and Co.

Human plasma and serum.—Human blood-bank plasma containing about 0·4 per cent sodium acid citrate and 0·6 per cent glucose was used. It was diluted to the required strength with 0·2 per cent sodium acid citrate, distributed in containers and stored at − 20°. For each experiment the contents of a container were allowed to thaw at room temperature and after use the residue was discarded. For serum, 25 ml. of the plasma was clotted with 0·1

ml. of sterile saturated calcium chloride solution, spun at 3500 r.p.m. for 30 min. and the serum stored at − 20°.

Bovine plasma fractions.—Armour Biochemicals.

Human plasma fractions.—Edinburgh and East of Scotland Blood Bank.

Egg albumin.—Armour Biochemicals.

Egg-white.—Fresh egg-white was mixed gently, aseptically with 2 vol. of saline in a slow-running electric mixer. The material was spun at 3000 r.p.m. for 30 min. and the clear supernatant stored at − 20°.

Histamine.—Histamine acid phosphate (Evans Medical Supplies Ltd.) was used: 3 mg. is equivalent to 1 mg. of histamine.

Anti-histamine.—Mepyramine maleate B.P. (" Anthisan ", May and Baker).

Sensitisation and challenge.—Unless otherwise stated, mice throughout were sensitised with 3 intraperitoneal injections at intervals of 7 days and challenged 7 days after the last sensitising injection with an intravenous injection; all injections were of 0·5 ml. The term sensitising dose means the total amount of antigen injected regardless of the number of injections.

Assessment of shock.—In the text shock is referred to as mild, severe, or fatal; in mild shock mice appear normal for about 5 min. after challenge then start to scratch the nose and ears vigorously; the face begins to swell, the face and ears become flushed, and the eyes watery and red-rimmed. The animals recover in 15 to 30 min. In severe shock these symptoms are followed within the next 5–10 min. by prostration, the animals moving only when disturbed; breathing is slow and deep and there is a marked fall in body temperature. They lie thus for a further 10 to 15 min., the tail and pads become cyanotic, and about 15 min. later begin to recover; after ½ hr. they may be sitting still hunched up, fur bristling, slowly returning to normal. In fatal shock, death usually occurs within 1 hr. of challenge, and is preceded by gasping breathing and convulsions at irregular intervals.

RESULTS

Sensitising route

The intraperitoneal, intravenous and subcutaneous routes for sensitisation were compared using bovine gamma globulin as antigen (Table I). The intraperitoneal and subcutaneous routes, almost equally effective, are superior to the intravenous route, particularly when the challenging dose is small.

TABLE I.—*A Comparison of 3 Routes of Sensitisation in the W-Swiss Mouse*

Sensitising* route.	Challenging dose (mg.).	Number of mice. Challenged.	Died.	Shocked. Severely.	Shocked. Mildly.	Unshocked.
Intraperitoneal	10	20	9	11	0	0
Intravenous	10	16	6	1	1	8
Subcutaneous	10	21	12	9	0	0
Intraperitoneal	1·0	21	4	13	4	0
Intravenous	1·0	14	1	4	5	4
Subcutaneous	1·0	22	3	14	3	2

* Mice were sensitised with 3 injections of a 2 per cent saline solution of bovine gamma globulin at intervals of 7 days; they were challenged intravenously with 0·5 ml. of a saline solution containing the stated quantity of antigen.

Challenging route

Again with bovine gamma globulin the same routes were investigated for challenging. The results show that the intravenous route is clearly the most effective; of the mice challenged intraperitoneally or subcutaneously less than half were shocked and those, in the main, mildly (Table II).

TABLE II.—*A Comparison of 3 Routes for Challenging Sensitised* W-Swiss Mice*

Challenging route.	Number of mice. Challenged.	Died.	Shocked. Severely.	Shocked. Mildly.	Unshocked.
Intraperitoneal	20	1	2	6	11
Intravenous	20	9	11	0	0
Subcutaneous	20	0	0	4	16

* Mice were sensitised with 3 intraperitoneal injections of a 2 per cent saline solution of bovine gamma globulin at intervals of 7 days; challenged with 0·5 ml. of the same solution.

Duration of sensitisation

Sensitisation was found to develop as early as 14 days after the first injection and to reach a peak 7 days later; the period of maximum sensitivity lasts about 4 weeks.

Effect of the number of sensitising injections.

The effect on sensitisation of fractionating a fixed amount of antigen was next examined; three antigens, egg, human and bovine albumin, were used. They were given in a single injection or as a series of fractionated doses, fifteen at most. None of the mice sensitised with the large single dose were shocked when challenged but as the number of sensitising injections increased so too did the incidence of severe and fatal shock. With egg albumin, of 9 mice given 15 sensitising injections, 7 were fatally shocked, the other 2 severely (Table III). The effect was not so marked with bovine albumin.

TABLE III.—*Effect on Sensitisation in the W-Swiss Mouse of Fractionating a Fixed Quantity of Antigen*

Antigen.	Number of injections.	Interval between injections. (days).	Antigen per injection. (mg.).	Number of mice. Challenged.	Died.	Shocked. Severely.	Shocked. Mildly.	Unshocked.
Egg albumin	1	—	30*	5	0	0	0	5
	2	11	15	8	0	1	2	5
	3	7	10	6	1	0	2	3
	6	3	5	8	4	4	0	0
	15	1	2	9	7	2	0	0
Bovine albumin	1	—	30	4	0	0	0	4
	1	—	30*	5	0	0	0	5
	2	11	15	2	0	0	1	1
	3	7	10	5	0	0	0	5
	6	3	5	6	2	1	1	2
	15	1	2	22	0	0	2	20
Human albumin	1	—	30	5	0	0	0	5
	1	—	30*	5	0	0	0	5
	2	11	15	3	0	0	0	3
	3	7	10	6	1	1	1	3
	6	3	5	8	2	3	1	1
	15	1	2	11	4	3	4	0

All mice except those marked * were challenged intravenously 21 days after the first sensitising injection with 0.5 ml. of a 2 per cent saline solution of the antigen.

* These groups were challenged 7 days after the sensitising injection.

Sensitisation by various proteins.

Human plasma, various sera, egg-white, and several purified proteins were compared as antigens on the basis of fatality rate and incidence of shock. Human gamma globulin and the albumins, egg, bovine and human, were all poor antigens, mice sensitised with 30 mg. and challenged with 10 mg. remaining unshocked. Bovine and horse serum were more active, mice sensitised with 0·4 ml. developing shock when challenged with 0·1 ml. Human serum and plasma, human and bovine fibrinogen, bovine gamma globulin, and egg-white were the most active.

An attempt to determine the minimum sensitising and challenging doses of these latter antigens was complicated by the finding that the doses were interdependent; that is to say, by increasing the sensitising dose it was possible to decrease the challenging dose—within limits—and *vice versa*. Hence the minimum sensitising dose was taken to mean the smallest dose (spread over 3 injections) which would sensitise at least 75 per cent of the mice in a group to a challenging injection of 10 mg.; conversely the minimum challenging dose was the smallest dose causing shock in mice sensitised with 10 mg.

The results (Table IV) show that the minimum sensitising and challenging doses of human plasma and serum were 0·1 to 0·01 ml. Of the purified proteins human fibrinogen was the most active with minimum sensitising and challenging doses of 0·05 mg. and 0·05–0·0005 mg. respectively; next came bovine fibrinogen (1·0–0·1 mg. in both cases), bovine gamma globulin (1·0–0·1 mg. and 10–1·0 mg. respectively) and egg-white (1·5 mg. and 1·5–0·15 mg. respectively).

TABLE IV.—*Minimum Sensitising and Challenging Doses of Various Antigens*

Antigen.	Dose.	Number of mice. Challenged.	Died.	Shocked. Severely.	Shocked. Mildly.	Unshocked.
*Minimum sensitising dose**						
Human fibrinogen	0·1 mg.	11	0	10	0	1
Bovine fibrinogen	1·0–0·1 mg.	24	9	13	0	2
Bovine gamma globulin	1·0–0·1 mg.	25	8	8	2	7
Egg-white	1·5 mg.	23	1	17	3	2
Human plasma	0·1–0·01 ml.	13	2	10	1	0
„ serum	0·1–0·01 ml.	10	3	5	2	0
*Minimum challenging dose***						
Human fibrinogen	0·1–0·001 mg.	16	0	14	2	0
Bovine fibrinogen	0·1 mg.	30	11	15	0	4
Bovine gamma globulin	10–1·0 mg.	310	130	122	32	26
Egg-white	1·5–0·15 mg.	32	6	18	6	2
Human plasma	0·1–0·01 ml.	27	9	14	1	3
„ serum	0·1–0·01 ml.	17	4	11	2	0

* Mice were challenged with 10 mg
** Mice were sensitised with 10 mg.

Comparison of the activity of egg fractions.

The minimum sensitising dose of unfiltered egg-white was found to be about 1·5 mg. In contrast 42 mg. of Seitz-filtered egg-white gave rise only to mild shock in all of 5 mice, and 40 mg. of egg albumin to severe shock in only 3 of 24

mice ; higher doses were not tested. Thus unfiltered egg-white is at least 28 times more active as an antigen than either filtered egg-white or egg albumin. The superior activity of unfiltered egg-white is clearly due to a protein fraction lost on filtration ; this fraction is also absent from crystallised egg albumin.

Prevention of anaphylactic shock by Anthisan.

The shock-preventing action in sensitised mice of an anti-histamine, Anthisan, was investigated in the following way. First an attempt was made to estimate the dose of Anthisan required to prevent histamine shock. This in turn entailed the estimation of the shock-inducing dose of histamine.

The effect of histamine given intravenously was tested on 42 mice. It was found that 0·15 mg. per g. (4·5 mg. per 30 g. mouse) or more killed instantly all 6 mice injected and that only one of 3 died instantly following the smaller dose of 0·14 mg. per g. With still smaller doses ranging from 0·13 to 0·0013 mg. per g. none of 33 mice died on injection but after 5–10 min. they developed symptoms similar to anaphylactic shock. The nose- and ear-scratching seen in the early stages were rare and even when the animal was prostrated, as in severe anaphylaxis, recovery, complete in 20–25 min., was more rapid. Only 2 died ; the convulsions and gasping breathing seen in anaphylactic shock were absent.

The dose of 2·5 mg. histamine, which invariably produced shock, was chosen as the dose against which to test the protective action of Anthisan. It was found that 0·5–2 mg. Anthisan given subcutaneously 15–30 min. beforehand gave complete protection.

In the experiment proper it was found that 2·5 mg. of Anthisan given subcutaneously 30–60 min. before challenge afforded almost complete protection against anaphylactic shock. The mice were sensitised with a potent antigen, bovine gamma globulin, and the challenging injection, 10 mg., was at least ten times the dose required to produce severe shock in sensitised control mice. Of 16 protected mice 3 developed shock ; one died.

DISCUSSION

In many respects—the existence of a latent period preceding the development of sensitisation, the need to give the challenging dose intravenously, the clinical picture, and the prevention of shock by an anti-histamine—anaphylaxis in mice resembles anaphylaxis in other animals excluding the guinea-pig which, in its extreme reaction, is probably unique. One point, the size of the sensitising and challenging doses, tended to set apart the reaction in mice from that in other animals. This seems to be resolved in the present work in which the doses are comparable to those used in other animals.

Of the antigens used, globulins appear to be more active than albumins. Human and bovine fibrinogen were the most active antigens, followed by bovine gamma globulin ; human gamma globulin, the least active, was an exception among the otherwise highly active globulins. The albumins, egg, human and bovine, were poorly active. Despite its content of extremely active fibrinogen, human plasma was no more active than human serum. Again, surprisingly, when human and bovine serum were compared human serum, with its poorly active albumin and gamma globulin, was more active than bovine serum with

its highly active gamma globulin. The activity of globulins other than the gamma fraction may explain these findings.

It should be pointed out, since at times they seem to be considered synonymous, that egg-white and egg albumin are not equally effective as antigens. Egg-white is about 28 times more active than egg albumin and its superior activity is clearly not due to its albumin content which is lower than that of the albumin solutions used ; the highly active fraction of egg-white is removed by Seitz-filtration.

Weiser *et al.* (1941) observed that in mice the same quantity of antigen was more effective spread over 4 doses. In the present work it was found that increasing the number of sensitising injections to 15 gave a higher degree of sensitisation with less active antigens. The degree of sensitisation appears to be directly related to the number of sensitising injections.

Mice, on the whole, particularly the W-Swiss, seem to tolerate large doses of histamine (Mayer and Brousseau, 1946 ; Perry and Darsie, 1946) although strains are known to differ in their resistance to histanine (Fink and Rothlauf, 1954). This has led Perry and Darsie (1946) to doubt the rôle of histamine release in anaphylactic shock in the mouse. In their view fatal amounts of histamine (or analogue) could hardly be liberated as the result of a challenging injection. Indirectly this view is supported by the present finding that increasing the challenging dose leads to increased mortality, but does not cause instant death as does a large dose of histamine. On the other hand, over a wide range of dosage the histamine effect closely simulates anaphylactic shock ; it is tempting to assume that the liberation of small amounts of histamine (or an analogue) is one of a series of reactions culminating in anaphylactic shock ; death may not be the direct result of histamine toxicity.

Whatever the rôle of histamine, anti-histamines are generally considered to be effective in preventing anaphylactic shock. In that respect anaphylactic shock in the mouse is no exception. The fact that anaphylactic shock in the mouse is prevented by an anti-histamine brings the phenomenon into line with that observed in other species.

SUMMARY

Anaphylactic shock was produced regularly in W-Swiss mice following three intraperitoneal sensitising injections at intervals of 7 days and an intravenous challenging injection 7 days after the last sensitising injection.

Mice were sensitised thus with human, bovine and horse serum, human plasma, egg-white, bovine fibrinogen and bovine gamma globulin. Human, bovine and egg albumin were poorer antigens and more sensitising injections were necessary.

Sensitisation, detectable 14 days after the first sensitising injection, is maximal from the third to the seventh week after the first sensitising injection and the degree of sensitisation is proportional to the number of sensitising injections.

An anti-histamine, mepyramine maleate (" Anthisan "), was found to protect mice from both histamine and anaphylactic shock.

I should like to take this opportunity of thanking Dr. I. R. W. Lominski of the Bacteriology Department, Western Infirmary and University, Glasgow, for his constant advice during the preparation of this paper.

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FACTORS INFLUENCING CHEMOTAXIS OF THE POLYMORPHONUCLEAR LEUCOCYTE

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ALTHOUGH it has long been known that the polymorphonuclear leucocyte is able to exhibit chemotaxis, there is very little information about the mechanism of the directional response and the factors which influence it. Delaunay, Lebrun and Barber (1951) reported that calcium, serum and complement were necessary for chemotaxis to occur in leucocytes and that other substances such as citrate, azide and fluoride could abolish the chemotactic response without impairing the motility of the cell.

In the present work some of the factors that are alleged to influence chemotaxis of the polymorph have been investigated.

EXPERIMENTAL

The method used for studying chemotaxis was the photographic trace technique devised by Harris (1953). Human polymorphs from a drop of blood obtained by finger-prick were made to adhere to a coverslip and were then washed three times with physiological saline in order to remove, as far as possible, any trace of serum. The cells were incorporated in a slide-coverslip preparation which was incubated at 37°, and their movement relative to a test object was recorded photographically. A chemotactic response was shown by a polarisation of the traces made by the leucocytes in the vicinity of the test object. The test object in the present experiments was a small clump of *Staphylococcus albus*.

RESULTS

Chemotaxis in solutions free from complement

Rabbit serum was heated for 30 minutes at 56° and was then shown to have lost its haemolytic complement activity. Chemotaxis of the polymorphs occurred readily in media containing such serum.

A volume of Hanks' physiological saline (Hanks, 1948) containing 2 g. per cent of albumin was made up, and was shown to be free from haemolytic complement activity. Chemotaxis occurred also in this medium. Since the heat-inactivated rabbit serum and the solution of albumin used in the two previous experiments may have had some complementary activity which was not detected by the haemolytic test system, a protein-free medium was tested. This consisted of Hanks' solution to which 10 g. per cent of polyvinylpyrrolidone had been added in order to increase the viscosity of the medium. Chemotaxis occurred in this medium also, the cells remaining alive for about 12 hours.

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Chemotaxis in solutions free from glucose

Hanks' solution was made up without glucose but with 10 g. per cent of polyvinylpyrrolidone. In this solution chemotaxis occurred, but the leucocytes remained alive for only about 4 hours instead of for 16 hours or more, as in serum-containing media.

Chemotaxis in solutions relatively free from calcium and magnesium ions

Delaunay *et al.* (1951) reported that when 10 mg. per ml. of sodium citrate were added to serum chemotaxis was inhibited. This inhibition was attributed to the removal of ionised calcium from the serum. In the present experiments an attempt was made to produce a medium as free from calcium and magnesium ions as possible.

Hanks' solution was made up without calcium and magnesium, and to this solution "Versene" was added in a concentration of 15 mg. per ml. This concentration of Versene was sufficient to chelate about twice the maximum stated calcium and magnesium impurities present in the "Analar" ingredients of the Hanks' solution. All glassware had been cleaned by prolonged treatment with acid. The polymorphs were washed repeatedly with physiological saline before being incorporated in the slide-coverslip preparation. In this medium containing Versene the movement of the cells was sluggish, but chemotaxis nevertheless occurred. With concentrations of Versene greater than 15 mg. per ml. chemotaxis did not occur, but the cells in such solutions were barely motile and died within an hour. The addition of calcium and/or magnesium to media containing toxic concentrations of Versene did not reduce the toxicity of these media. It was therefore concluded that this toxicity was due to the Versene itself and not to any effect it might have had in chelating divalent cations. The addition to the medium of sodium citrate in a concentration of 10 mg. per ml., as used by Delaunay *et al.* (1951), did not impair the motility of the cells or their ability to exhibit chemotaxis.

Survival of the cells in coverslip chambers

In the slide-coverslip technique used in the present experiments the polymorphs moved in a relatively small volume (about 0·02 ml.) of test solution. Traces of serum which might have remained adherent to the cells, despite repeated washing in saline, would undergo less dilution in this small volume of medium than under conditions where a greater volume of medium could be used. Experiments were therefore carried out with coverslip chambers having a capacity of about 0·5 ml. The polymorphs for these experiments were obtained from the peritoneal cavity of a rabbit one day after the injection of a solution of soluble starch. The cells were washed repeatedly by centrifugation in saline and introduced into the chambers containing the various test solutions mentioned above. For technical reasons demonstrations of chemotaxis could not be made in these larger volumes of fluid, but the motility and survival of the cells could be studied and a comparison could be made between the behaviour of the cells in the slide-coverslip preparations and their behaviour in the larger volumes of medium. It was found that the cells survived in the chambers several hours longer than in the slide-coverslip preparations but in other respects their behaviour was similar. It therefore seemed improbable that the survival and movement of the cells in the experiments

carried out in protein-free media and media deficient in glucose or calcium and magnesium ions, were due to the presence of retained serum.

The effect of changes in the tonicity of the medium

Hypotonic media were made by adding to the Hanks' solution increasing amounts of distilled water ; hypertonic media were made by adding to the Hanks' solution increasing amounts of one of the following substances : sodium chloride, mannitol, urea and sucrose. It was found that the polymorph remained motile and exhibited chemotaxis in hypotonic solutions until a fall in tonicity of about 125 milliosmoles was produced. Below this level the cells survived for only a few minutes and chemotaxis could not be demonstrated. The degree of hypertonicity which the cells were able to withstand varied with the substance added. With urea the cells withstood an increase of 500 milliosmoles ; with glucose 200 milliosmoles ; with mannitol, sucrose and sodium chloride 100 to 125 milliosmoles.

Negative chemotaxis

McCutcheon, Coman and Dixon (1939) reported that aluminium silicate was negatively chemotactic to polymorphs in the sense that a clump of this substance caused the cells in its vicinity to move actively away from it. In the present experiments it was not possible to demonstrate negative chemotaxis with aluminium silicate. The leucocytes appeared to be quite indifferent to this substance, and particles of it were phagocytosed by them when random movement brought them in contact with such particles.

The effects of some chemical agents

Delaunay *et al.* (1951) reported that the following substances in the concentrations mentioned could abolish chemotaxis without impairing the motility of the cell : sodium cyanide (0·011 M), sodium azide (0·143 M), sodium fluoride (0·25 M), cysteine (0·100 M), malonic acid (0·143 M), sodium pyrophosphate (0·005 M) and sodium bisulphite (0·125 M). In the present work sodium fluoride, sodium azide, cysteine, malonic acid and sodium bisulphite in these concentrations were found to kill the cells almost immediately. However, in solutions containing 0·011M sodium cyanide and 0·005 M sodium pyrophosphate, the cells remained motile for about 10 hours and chemotaxis could be demonstrated.

Chemotaxis occurred readily in solutions containing sodium azide, sodium fluoride or sodium cyanide at final concentrations of 0·001 M and sodium azide or malonic acid at final concentrations of 0·005 M.

DISCUSSION

These experiments show that chemotaxis by polymorphs can occur in the absence of complement, serum or glucose, and in solutions relatively free from ionised calcium or magnesium.

These cells are able to withstand large increases or decreases in the tonicity of the ambient medium. The degree of hypertonicity which they will tolerate varies with the substance added. It appears that much greater increases in tonicity can be withstood when these are produced by substances which enter

the cell freely, such as urea, than by substances which do not, such as sucrose or mannitol.

The ability of polymorphs to survive and remain motile for considerable periods in solutions devoid of any known source of energy suggests that these cells may have an intrinsic energy store. The fact that the cells remained motile for many hours in solutions containing 0·001 and even 0·011 M sodium cyanide or 0·005 M sodium azide suggests that their survival is not acutely dependent upon a supply of oxygen.

The suggestion that chemotaxis can be inhibited without impairing the motility of the cell is not supported by the present experiments. Chemotaxis was found to be suppressed only when motility was suppressed, that is, under conditions toxic for the cell.

The discrepancies between the results of the present experiments and those reported by Delaunay *et al.* (1951) may be due, in part, to differences in technique. The photomicrographic trace technique used here records the actual movement of leucocytes, and chemotaxis is said to have occurred when a clear cut directional response is recorded. In Delaunay's experiments the adherence of leucocytes to grains of starch was considered evidence of chemotaxis. While this adherence may under certain circumstances indicate that chemotaxis has occurred, the phenomenon would appear to involve a great deal more than chemotaxis, as has been recently shown by Nelson and Lebrun (1956). A discussion of the criteria for demonstrating chemotaxis has been given elsewhere (Harris, 1954).

SUMMARY

Chemotaxis of polymorphonuclear leucocytes was demonstrated in the absence of complement, serum or glucose, and in solutions with no detectable concentration of ionised calcium or magnesium. It was not found possible to inhibit chemotaxis without at the same time impairing the motility of the cell. The ability of the polymorph to withstand increases and decreases in the tonicity of the ambient medium was examined.

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THE BASIS OF VIRULENCE IN *PASTEURELLA PESTIS*: AN ANTIGEN DETERMINING VIRULENCE

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In previous studies (Burrows and Bacon, 1956) it was shown that virulent strains of *Pasteurella pestis* could be differentiated from avirulent strains (including "protective" avirulent strains capable of producing envelope antigen and active immunity) by the ability of the former to develop resistance to phagocytosis by mouse polymorphonuclear leucocytes, under defined conditions *in vitro*. Apart from this important property virulent and protective avirulent strains were indistinguishable *in vitro*. It was evident that the ability to develop resistance to phagocytosis, which occurred in the absence of visibly demonstrable capsulation (or "envelope"), was essential for the manifestation of high virulence.

The work reported here was designed to test for the presence in phagocytosis-resistant virulent organisms of an antigen conferring resistance in the absence of visibly demonstrable capsulation, and for its absence in avirulent organisms not capable of developing resistance. If resistance depended on the action of an antigenic component, anti-sera produced against resistant organisms should effectively neutralise its action and render resistant-organisms sensitive to phagocytosis. In contrast antisera produced against sensitive virulent or avirulent organisms should be ineffective in this respect. The experiments support the hypothesis.

MATERIALS AND METHODS

Strains and suspensions

As in previous studies the fully virulent strain MP6 and the protective avirulent strain TS were mostly used. Details of their maintenance, and of the preparation of stock suspensions and cultures were as previously described (Burrows and Bacon, 1956).

Vaccines

All vaccines were used in the living state. They will be referred to throughout by the abbreviations M/S, M/R, M/M and T/'R' which are defined below.

M/S = Phagocytosis-sensitive virulent organisms :—

MP6 organisms harvested from tryptic meat agar (TMA) slopes, which had been grown for 17 hr. at 28°, inoculated into tryptic meat broth (TMB) pH 7 to a concentration of 2×10^8 organisms per ml. and then incubated with gentle rotation for 3 hr. at 28°. Virulent organisms treated in this way were not visibly capsulated, as judged by the India ink method of Rowland (1914), and were highly sensitive to phagocytosis.

M/R = Phagocytosis-resistant virulent organisms :—

MP6 organisms grown and treated precisely as M/S above except that the temperature of incubation with rotation in TMB was 37° instead of 28°. Virulent organisms so treated were not visibly capsulated but were resistant to phagocytosis.

M/M = Phagocytosis-resistant virulent organisms grown *in vivo* :—

Previous studies had shown that virulent organisms derived from mice moribund from virulent infection were very highly resistant to phagocytosis in tests *in vitro*. We anticipated that such organisms would possess the presumed antigen conferring resistance in abundance and would evoke anti-sera with high specific antibody content. They were therefore included in this series of vaccines.

To obtain organisms in this condition 10 mice were injected intraperitoneally each with 1×10^7 M/R and 10 similarly injected each with 2×10^7 M/R. From the two groups 8–14 moribund animals were available 16 hr. after injection, the remainder being either dead or apparently well. The moribund animals were injected i.p. each with 1 ml. heparinised saline, killed by cervical dislocation and the peritoneal fluids containing organisms in suspension recovered with a pipette. The pooled suspensions were centrifuged lightly, to deposit polymorphs and erythrocytes, and the supernatants vigorously centrifuged to deposit organisms. Each moribund mouse yielded *ca.* 2×10^9 organisms. Virulent organisms obtained in this manner were visibly well capsulated and were highly resistant to phagocytosis.

T/' R ' = Phagocytosis-sensitive avirulent organisms :—

TS organisms grown and treated precisely as M/R above. The abbreviation ' R ' is used to indicate sensitivity to phagocytosis and that the immediate history for the growth of T/' R ' was that of M/R and not that of M/S. Avirulent organisms so treated were not visibly capsulated and were highly sensitive to phagocytosis. All organisms after treatment as above were sedimented by centrifugation and re-suspended in phosphate buffer pH 7 so that 0·2 ml. volumes contained the desired vaccine dose for animal injection, or to the density required for other purposes.

Production of anti-sera

Anti-sera were produced in mice and in rabbits. Before using live virulent organisms as vaccines it was necessary to pre-immunise normal animals. Live purine-dependent (and thus avirulent) organisms of strain M1 (derived from MP6 (Burrows and Bacon, 1954*a*, *b*)) were used for this purpose. Organisms used for pre-immunisation were treated similarly to those used for the main vaccines, excepting that for animals later to receive M/M the pre-immunising vaccines were the same as those given to the M/R group. To maintain comparability, although not necessary for safety, animals which were to receive T/' R ' were pre-immunised with the purine-dependent strain T3 derived from TS.

Four rabbits (1·8–2·2 kg.) and 80 mice (Porton strain, 18–22 g.) were used for each vaccine. Injections were given intraperitoneally into mice and subcutaneously into rabbits at about 7-day intervals. The doses of organisms given are shown in Table I.

TABLE I.—*Scheme of Immunisation*

		Organisms for: Pre-immunisation. M1/S M1/R M1/R or T3/'R'			Main vaccines. M/S M/R M/M or T/'R'						
Dose No.		1	2	3	4	5	6	7	8	9	10 to 17
Organisms per dose	Mice	10^6	10^7	10^7	10^3	10^4	10^5	10^6	10^7	10^8	10^6
	Rabbits	10^8	10^9	10^9	10^7	10^8	10^9	10^9	10^9	10^9	2×10^9

Rabbits were bled from the ear vein seven days after doses 8, 12, 15 and 17. Ten mice, chosen at random from each group of 80, were anaesthetised with chloroform seven days after doses 12 and 15, bled from the heart and bleedings pooled. The remaining mice were similarly bled seven days after dose 17. For the majority of experiments the sera from successive bleedings of all animals of any one group were pooled for use. Serum from a rabbit hyper-immunised with live M/R over the course of a year was used for certain experiments. All sera were stored frozen without added preservative.

Polymorph suspensions

Mouse polymorphs were used throughout, unless otherwise stated. These were collected, suspended in phosphate-gelatin-acetate medium (PGA) and manipulated as previously described (Burrows and Bacon, 1956).

Tests for ability of sera to render resistant-organisms sensitive to phagocytosis

This property will be referred to as ability to sensitise. To 0·4 ml. amounts of sera contained in waxed tubes, and usually at a dilution of 1/2 in PGA, was added 0·05 ml. of a suspension of M/R containing 1×10^{10} organisms per ml. After 10 min. at 37° the tubes were cooled to 20° and 0·4 ml. of a suspension containing *ca.* 3×10^{7} mouse polymorphs per ml. added to each. Without delay the contents were thoroughly mixed and distributed in 0·2 ml. volumes to each of 3 waxed tubes. All tubes were then rotated for 30 min. at 37°, rapidly chilled in iced water and films prepared from each. Phagocytosis was estimated as previously described (Burrows and Bacon, 1956). Only the means of triplicate determinations of the numbers of organisms ingested per 100 polymorphs are given in the Tables, because no additional information is provided by quoting the percentage of polymorphs containing ingested organisms.

Fraction 1 (*envelope*) *antibody content of sera*

This was determined by adding 0·1 ml. of a saline solution of purified fraction 1 (Baker, Sommer, Foster, Meyer and Meyer, 1952) containing 1 mg. fraction 1 per ml., to 1 ml. of the serum under test and incubating for 1 hr. at 37°. The resulting turbidity was referred to a curve obtained by adding equal amounts of fraction 1 to known dilutions of a specific rabbit anti-serum taken as a standard. In the Tables the anti-fraction 1 content of sera is expressed relative to that of the standard serum which was arbitrarily assigned a value of 100. In this paper the terms " capsule " and " envelope " are treated as synonymous.

Absorption of sera

To absorb sera with living organisms, to each 1 ml. of serum was added 0·33 ml. of a PGA suspension containing 1×10^{10} organisms and the mixture incubated for 3 hr. at 37° with rotation. The process was repeated with 2 further equal additions of organisms. After the third absorption the serum (now at a dilution of 1/2 in PGA) was centrifuged and filtered through a millipore membrane.

For absorption with acetone-dried products, organisms were grown in the manner described for the production of vaccine and dried with acetone by the method of Baker *et al.*, (1952). To each 1 ml. of serum 100 mg. of acetone-dried organisms were added, the mixture incubated at 37° for 3 hr., then vigorously centrifuged. To absorb sera with fraction 1, 2·5 mg. of purified antigen was added to each 1 ml. of serum and the mixture treated as for absorption with acetone-dried organisms.

RESULTS

The majority of mice receiving live T/' R ', M/S or M/M vaccines tolerated the injected doses well. Those receiving M/R tolerated dosages up to 10^{6} but many failed to survive a dose of 10^{7} resistant virulent organisms ; 36 of the 80 animals in this group died. Post-mortem examination revealed large numbers of organisms in agglutinated masses in the peritoneal cavity. Doses of 10^{7} T/' R ', M/S and M/M resulted in 0, 8 and 6 deaths respectively. A feature of interest was the health and vigour shown by mice in the M/M group. This was reflected in the average weights of animals measured after dose 12 : M/M group 42 g., M/R 34 g., M/S 31 g. and T/' R ' 34 g. All rabbits tolerated immunisation well.

In experiments to determine the comparative abilities of sera to sensitise M/R and M/M unpredictable variation occurred from experiment to experiment both in the extent of ingestion of untreated organisms and in the extent of ingestion of organisms treated with sensitising sera. This variation probably resulted in

part from the unavoidable use of fresh suspensions of organisms and of polymorphs for each experiment performed on different days. While the absolute sensitising ability of any one serum varied considerably between experiments, the relative sensitising abilities of different sera showed little variation in repeat determinations. Table II illustrates the variation experienced in a number of assessments of the ability of a rabbit hyperimmune anti-M/R serum to sensitise M/R and M/M. It also shows the relatively higher resistance of M/M, compared with M/R, and the high ability of this serum to sensitise both types of organism. Wherever possible the relative sensitising values of sera were compared within one experiment and unless otherwise indicated only results so obtained are quoted in subsequent Tables.

TABLE II.—*Variation in Phagocytosis of M/R and M/M after Sensitising with Normal or with Anti-M/R Rabbit Sera*

		Phagocytosis.					
		Normal serum treated.			Immune serum treated.		
Organism.	No of Expts.	Lowest.	Highest.	Mean.	Lowest.	Highest.	Mean.
M/R	17	47	765	263	555	2815	1313
M/M	15	4	269	54	677	2198	1290

Phagocytosis = numbers of organisms ingested per 100 mouse polymorphs. Immune serum derived from rabbit hyperimmunised with live M/R for one year.

The comparative abilities of antisera produced in mice to render resistant-organisms sensitive to phagocytosis are shown in Table III.

TABLE III.—*Comparative Abilities of Anti-sera Produced in Mice to Sensitise M/R and M/M*

Mouse serum.		Phagocytosis of M/R.	Phagocytosis of M/M.	Relative anti-fraction 1 content.
Normal		315	17	0
Against living	T' R '	668	258	31
	M/S	679	336	20
	M/R	1879	585	30
	M/M	884	444	57

Phagocytosis = numbers of organisms ingested per 100 mouse polymorphs. All sera used at 1/4 final concentration in test system.

All mouse anti-sera were found to have some ability to sensitise M/R and M/M; the order for decreasing ability to sensitise M/R being sera M/R, M/M, M/S, T/' R '. Serum produced against M/R was particularly effective in rendering M/R sensitive to ingestion. Sera produced against M/S and T/' R ' did not materially differ in their sensitising properties. The anticipated high quality of anti-sera produced against highly resistant virulent organisms grown *in vivo* (M/M) was not obtained. The abilities of anti-sera to sensitise either type of resistant organism were not correlated with their content of fraction 1 antibody. The highest anti-fraction 1 content, as might be expected, appeared in anti-sera against M/M. It will be remembered that M/M organisms were visibly capsulated while T/' R ', M/S and M/R were not.

We concluded from these results that the resistance to phagocytosis of M/R was determined primarily by an antigen other than fraction 1, and that this resistance-determining antigen was present in greater amounts, or in a more highly immunogenic state in M/R than in other vaccines. Although living organisms were used for all vaccines, anti-sera produced against virulent organisms in the /S, /R and /M conditions were quantitatively distinguishable. This suggests that live vaccines injected into pre-immunised animals did not materially change *in vivo* from the antigenic state in which they were injected. From this it follows that they did not actively metabolise or multiply. If this were not so M/S *in vivo* would change, within a few hours, to M/R and eventually to M/M and sera produced against M/S, M/R and M/M would be quantitatively and qualitatively alike, which is not so. In some earlier experiments, in which mice were given a less intensive course of immunisation, anti-sera against T/' R ' and M/R both showed low sensitising ability towards M/R, suggesting that the material conferring resistance acts as a poor antigen in the mouse.

The comparative abilities of anti-sera produced in rabbits to sensitise M/R and M/M are illustrated in Table IV.

TABLE IV.—*Comparative Abilities of Anti-sera Produced in Rabbits to Sensitise M/R and M/M*

Rabbit serum.		Phagocytosis of M/R.		Phagocytosis of M/M.	Relative anti-fraction 1 content.
Normal		275	(120)	81	0
Against living	T/' R '	431	(346)	397	21
	M/S	820	(627)	942	23
	M/R	951	(1637)	1332	22
	M/M	327	(1141)	430	70

Phagocytosis = numbers of organisms ingested per 100 mouse polymorphs. Bracketed figures = ingested organisms per 100 rabbit polymorphs. Sera used at 1/4 final concentration in phagocytic system.

As with anti-sera produced in mice, those from rabbits all showed some ability to sensitise both M/R and M/M, anti-sera against M/S and M/R being superior to those against T/' R ' and M/M. There was no clear superiority of anti-sera against M/R over those against M/S in ability to sensitise M/R. However, it will be shown later that rabbit anti-M/R serum in the unabsorbed condition did not manifest its full sensitising ability for M/R when tested with mouse polymorphs. Similarly rabbit anti-M/M sera contained antibodies to mouse components, which contaminated the vaccine, and which had an adverse effect when these sera were used in phagocytic systems employing mouse polymorphs. In systems employing rabbit polymorphs the relative abilities of anti-sera produced in rabbits to sensitise M/R were very similar to those of anti-sera produced in mice and tested in systems employing mouse polymorphs, *i.e.*, the decreasing order of ability to sensitise again being sera M/R, M/M, M/S, T/' R '. As with anti-sera produced in mice, ability to sensitise was not correlated with anti-fraction 1 content.

Anti-sera against all four vaccines contained fraction 1 antibody which was removable by heavy absorption with any of the live vaccines. All vaccines must therefore have had fraction 1 as a surface antigen although only M/M had it in sufficient quantity to be observed as a capsule. In sera containing fraction 1

antibody M/M agglutinated into large compact aggregates (observed microscopically) whereas T/' R ', M/S and M/R agglutinated in considerably smaller, less compact and more easily disrupted aggregates. A possible explanation of these observations is that fraction 1 in T/' R ', M/S and M/R is in insufficient quantity to provide full surface coverage to these organisms and is distributed in small isolated islands on the bacterial surface. In M/M this antigen is in abundance giving full surface coverage, visible capsulation and " solid " agglutination with fraction 1 antibody. Whether this capsule consists exclusively of fraction 1 has not been proved. (Although this antigen extracted by mild chemical methods is readily soluble in saline, M/M stored in saline at 4° over many months retained full visible capsulation over this period, suggesting that fraction 1 in the intact capsule exists in a bound relatively insoluble state).

Comparisons of the sensitising abilities of a high titre anti-serum produced in rabbits against a purified preparation of fraction 1, and of rabbit anti-M/R serum showed the former to be relatively deficient in ability to sensitise M/R. Towards M/M however, the two anti-sera had similar high sensitising ability. Absorption of anti-M/R serum with fraction 1, sufficient to leave excess fraction 1 in the absorbed serum, did not greatly reduce the sensitising ability of the serum for M/R but removed ability to agglutinate and sensitise M/M. Similarly heavy absorption of anti-M/R serum with live T/' R ', which removed all fraction 1 antibody and also ability to agglutinate heavily capsulated M/M, did not materially affect its ability to sensitise M/R but again removed all ability to sensitise M/M. These results are presented in Table V.

TABLE V.—*Effect of Removal of Fraction* 1 *Antibody on Ability of Anti-sera to Sensitise M/R and M/M*

Expt.	Rabbit serum.	Absorbed with.	Phagocytosis of M/R.	Phagocytosis of M/M.
1	Normal	—	313	18
	Anti-M/R†	—	1181	604
	„ „	Fraction 1*	929	10
2	Normal	—	342	9
	Anti-M/R†	—	1478	933
	Anti-F 1	—	462	607
3	Normal	—	157	4
	Anti-M/R†	—	1747	995
	„ „	T/' R '**	1669	15

* Serum absorbed with 2·5 mg. purified fraction 1 per ml. serum.
** Serum absorbed thrice, each with 1×10^{10} organisms per ml. serum, for 3 hr. at 37°.
† Rabbit hyperimmunised with live M/R over a period of a year.
Phagocytosis = numbers of organisms ingested per 100 polymorphs.
All sera used at 1/4 final concentration in test system. Anti-fraction 1 serum contained merthiolate 0·01 per cent, which did not interfere in phagocytic tests.

From several experiments of this nature we concluded that the resistance to phagocytosis shown by M/R resulted from the action of an antigen other than fraction 1, whereas the resistance shown by M/M was determined primarily by its complement of fraction 1 antigen.

It would appear, for effective sensitisation of M/M, that either a high concentration of fraction 1 antibody (as in specific serum) or lower concentrations plus

another factor (as in anti-M/R serum) is required. The additional factor appears to be in minimum amount in anti-T/' R ' serum and maximum in anti-M/R serum. It is apparently ineffective in the complete absence of fraction 1 antibody.

While heavy absorption of rabbit anti-M/R serum with live T/' R ' failed to remove sensitising ability for M/R it was anticipated that like absorption with live M/R would do so. However, it was found that heavy absorption with M/R considerably enhanced sensitising ability for M/R when the absorbed serum was tested in a mouse polymorph system but reduced sensitising ability when tested in a rabbit polymorph system. Similar enhancement of activity was not obtained when rabbit anti-M/R serum was absorbed with T/' R ', M/S or M/M or when their anti-sera were absorbed with M/R. This unexpected enhancement of activity has not yet been satisfactorily explained but could conceivably be due to the removal from rabbit anti-M/R serum of an antibody having an adverse effect on mouse polymorphs, or on some other component of the phagocytic system. This conception might imply that M/R possesses an antigen which is serologically related to a component of the mouse. Attempts to prove this have so far failed. Alternative explanations are discussed later.

The effects of various absorptions of rabbit anti-sera on their ability to sensitise M/R are presented graphically in Fig. 1. Owing to the lack of precision of the

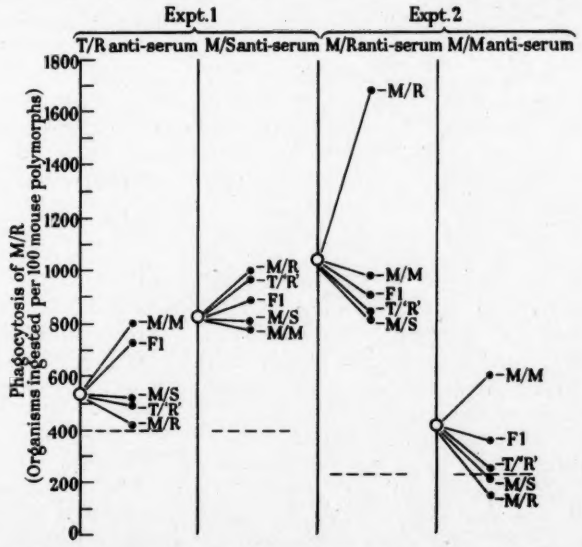


FIG. 1.—Effects of various absorptions on ability of rabbit sera to sensitise M/R. Sera absorbed thrice for 3 hr. at 37° using 1×10^{10} organisms per ml. serum for each absorption, or absorbed once with 2·5 mg. fraction 1 per ml. serum. ○ = Unabsorbed serum. ● = Absorbed serum. Phagocytosis in normal rabbit serum shown thus - - - - -. (T/R in heading of left hand column should be T/'R').

phagocytic test we do not feel justified in drawing firm conclusions of the effects of absorptions which resulted in small changes in sensitising ability. In repeat experiments such absorptions resulted in either no apparent change, slight

decrease, or slight increase in sensitising ability. The main conclusions drawn with confidence from this series of absorptions were first that, towards M/R, complete absorption with fraction 1 did not materially decrease sensitising ability, second that absorption of anti-T/' R ' serum with M/R removed sensitising ability and third that absorption of anti-M/R serum with M/R materially enhanced sensitising ability.

Antigenic analyses of vaccines by agar diffusion precipitation technique (Ouchterlony, 1949, 1953).

Comparisons of the sensitising abilities of anti-sera towards M/R supported the hypothesis that the resistance of M/R was mediated by an antigenic factor and that antibody to this factor was present in more effective amount in anti-sera prepared against M/R than in anti-sera prepared against the other vaccines. For ease of description we will call this factor V-antigen on the assumptions that a single antigen determines resistance to phagocytosis in M/R and that T/' R ' and M/S do not contain effective amounts of this antigen.

In attempts to demonstrate V-antigen visually we compared the patterns of antigen-antibody lines of precipitation formed when the different vaccines were diffused in agar against their homologous sera, with the pattern produced when M/R was diffused against rabbit anti-M/R serum. Our technique was essentially that of Crumpton and Davies (1956). However, the multiplicity of lines (12–18) obtained when living organisms were placed in the antigen reservoirs frequently made impossible the identification of every line with sufficient certainty to state that this or that line was absent from particular systems. All well-defined lines of precipitation appeared to be common to all vaccines. Other less well-defined lines, including therefore that corresponding with V-antigen, either were obscured by stronger lines or were insufficiently distinct to be observed to join, and be identified with, those present in the standard M/R-anti-M/R pattern. Increasing the distance between antigen and anti-serum reservoirs increased the resolution of strong lines but resulted in loss of weaker ones ; decreasing the distance decreased resolution so that the weaker became obscured by the stronger. Sera heavily absorbed with living organisms still retained detectable amounts of the majority of their various antibodies (except that to fraction 1) and particularly those giving strong precipitin lines. The use of such sera therefore did not aid attempts to recognise the presence of V-antigen.

In contrast with the absorption of anti-sera by living organisms, heavy absorption with acetone-dried preparations of our vaccines served to remove completely all antibodies to antigens present in such dried preparations used for absorption. It was therefore possible to absorb rabbit anti-M/R serum with acetone-dried T/' R ' and make it specific for antigens present in M/R and absent from T/' R '; among these would be the presumed V-antigen. It was thus found that rabbit anti-M/R serum absorbed with acetone-dried T/' R ' gave a single well-defined line of precipitation when diffused against live M/R, a weak line against live M/M but did not give this line when diffused against live M/S or T/' R '. This absorbed serum, despite large excess of many antigens, retained high ability to sensitise M/R. Equal absorption of rabbit anti-M/R serum with acetone-dried M/R removed all ability to sensitise M/R and removed all ability to give lines of precipitation with any of the live vaccines.

We thus had presumptive evidence that the line appearing when M/R was diffused against rabbit anti-M/R serum heavily absorbed with acetone-dried T/' R ' resulted from the union of V-antigen with its specific antibody. V-antigen was not detectable in M/S by the diffusion method but became readily detectable when such organisms were converted to the M/R state by incubation in TMB for 3 hr. at 37°. V-antigen was not produced when M/S was similarly incubated in CCY medium but was produced when this medium was supplemented with glucose. Previous studies had shown that CCY medium supplemented with glucose was effective for the development of resistance whereas unsupplemented medium was not (Burrows and Bacon, 1956).

V-antibody, readily detectable in rabbit anti-M/R serum as described above was not found in similarly absorbed rabbit or mouse anti-T/' R ' or anti-M/S sera. It was detectable in small amount in rabbit anti-M/M serum. In mouse anti-M/R and anti-M/M sera V-antibody was considerably less abundant than in the corresponding rabbit anti-sera.

The ability to become resistant to phagocytosis in the absence of visible capsulation characterised all virulent strains tested (Burrows, 1955). It follows therefore that, if the antigen conferring resistance (V-antigen) had been correctly identified all strains capable of developing resistance and being fully, or potentially, virulent should produce V-antigen under conditions suitable for the development of resistance. Conversely strains which are avirulent and incapable of developing resistance should, under the same conditions, fail to produce V-antigen.

Accordingly a number of virulent, potentially virulent, and avirulent strains, chosen randomly from a culture collection, were treated as described for the production of M/R and diffused against rabbit anti-M/R serum, rendered apparently specific for V-antibody by heavy absorption with dried T/' R '. As previously reported (Burrows, 1956) a line of precipitation identifiable with that given by M/R was clearly produced by the virulent strains Yokohama, Shasta, F9581, L36, L37 and by the strains M1 and M7, known to be potentially virulent. (All these strains were capable of developing resistance.) No trace of precipitation was given by the avirulent strains TS, A1122, 14, Java, Soemedang or 53H1 ; none of these was capable of developing resistance. A culture of strain 139L, which was presumed to be virulent, failed to give V-antigen line. When checked it was found incapable of developing resistance and was avirulent for mice at doses from 10^1 to 10^5 organisms per mouse. One avirulent strain tested, EV 76 of Girard and Robic (1936), was unique in that it produced V-antigen as readily as fully virulent strains. This strain, which has been used extensively as a living vaccine for plague prophylaxis in humans, was included in this survey because it had been reported to possess a " residual degree of virulence " (Girard and Radaody-Ralarosy, 1940). It thus differs in an important respect from the other widely used human vaccine strain Tjiwidej S (TS). Under conditions suitable for the conversion of M/S to M/R strain EV 76 develops resistance to phagocytosis whereas TS does not. We have also found strain EV 76 to be unique, among those we have examined nutritionally, in consisting predominantly of organisms which are dependent on an exogenous supply of uracil for growth. However, this uracil dependence does not appear to contribute to the avirulence of this organism.

It is clear that M/R differs from M/S and from T/' R ' in possessing demonstrable amounts of V-antigen. More recently, and so far in preliminary manner, we have observed that, on incubation in TMB for periods in excess of that required for

maximum development of resistance, M/R produces another antigen which further distinguishes it from M/S and T/' R '. This second antigen (designated by the letter W) has been detected only in strains capable of producing V. Both V- and W-antigens are freely soluble products and are readily detectable in supernatant fluid from cultures incubated for 6 hr. under conditions described for the production of M/R organisms. Lines of precipitation corresponding with V- and W-antigens are shown in Fig. 2. This illustrates their production by the virulent strains MP6, Yokohama, Shasta, F9581 and by the avirulent EV76 and not by the avirulent TS, A1122, Java and Soemedang.

DISCUSSION

It is apparent that at least two types of resistance to phagocytosis can occur in *P. pestis*; one associated with the presence of visible capsulation of the organisms, the other occurring in the absence of visible capsulation. Organisms showing the former type of resistance are compactly agglutinated and rendered sensitive to phagocytosis by treatment with antiserum containing fraction 1 antibody; when this antibody is removed by absorption the serum then fails to agglutinate capsulated organisms or render them sensitive to phagocytosis. Virulent organisms derived from moribund mice exhibit resistance of this first type. Avirulent organisms with the potentiality for fraction 1 antigen synthesis can show this type of resistance when recovered from mice moribund from massive avirulent infection (Burrows, unpublished data).

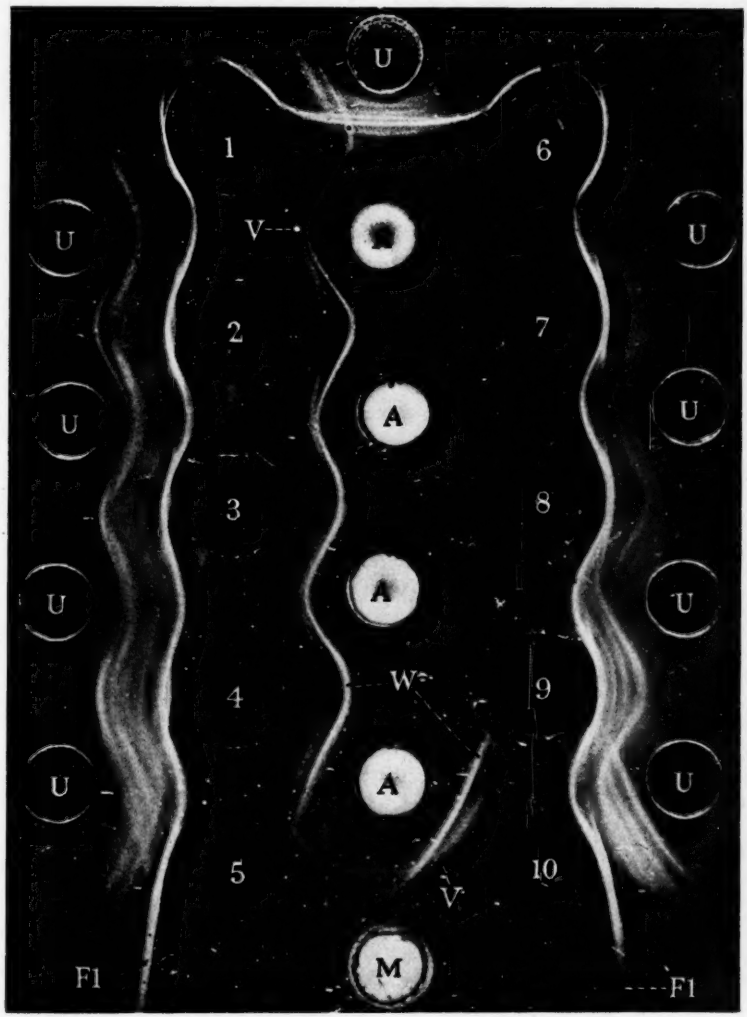
It is with the second type of resistance, which occurs in the absence of visible capsulation, that we have been mainly concerned. Organisms in this resistant state are not rendered sensitive to phagocytosis by the action of fraction 1 antibody. Anti-sera having high ability to sensitise these organisms retain this property after absorption with fraction 1. Such sera are produced in rabbits and in mice when organisms in this resistant condition are used as live vaccines. But in this respect sensitive virulent organisms grown *in vitro*, resistant virulent organisms derived from moribund mice, or avirulent organisms, are very poor antigenically. Among the representative strains examined the ability to develop resistance in the absence of visible capsulation is confined to fully, or potentially, virulent strains and to the avirulent strain EV76. (In this context potentially virulent strains are those, which we have selected from our representative virulent strain MP6, showing loss of virulence and to which full virulent behaviour can be restored by specific treatment of the host. An example is the purine-dependent avirulent strain M1 which behaves as a virulent strain in mice which have been injected

EXPLANATION OF PLATE

FIG. 2.—V and W antigen-antibody lines of precipitation. Petri dish containing 12 ml. saline agar with reservoirs. Reservoirs U contain rabbit anti-serum against live M/R. Reservoirs A contain the same serum absorbed with acetone-dried TS grown at 37°. Reservoir M contains live M/R organisms. Reservoirs 1 to 10 contain supernatants of cultures of different strains grown for 17 hr. in tryptic meat broth at 37° with rotation.

Virulent strains.—1. MP6. 2. Yokohama. 3. Shasta. 4. F9581. *Avirulent strains.*—5. TS. 6. TS. 7. A1122. 8. Java. 9. Soemedang. 10. EV76. V and W antigens restricted to virulent strains and EV76.

Fraction 1 line (F1) results mainly from diffusion of excess antigen from reservoirs containing absorbed serum. Avirulent strains, other than EV76 and Java, give a sharp thin line of precipitation which does not correspond with either V or W.



Burrows and Bacon.

with purines). This second type of resistance is attained when sensitive organisms are incubated in tryptic meat broth at 37° for 3 hr. The change from sensitivity to resistance to phagocytosis, is accompanied by the production of a specific antigen (V). This antigen has not been found in sensitive virulent or avirulent organisms ; it seems to be confined solely to those strains capable of developing resistance just described. So far as has been tested strains of *P. pestis* can therefore be divided into two groups on the basis of ability or inability to produce V-antigen. All fully or potentially virulent strains and EV76 produce V-antigen ; all protective avirulent strains except EV76 fail to produce it. Strains would be grouped in precisely the same way on the basis of ability or inability to develop resistance to phagocytosis in the absence of visible capsulation. On this evidence it would be reasonable to conclude that V-antigen was primarily responsible for this type of resistance. However, we have now observed that the members of the two groups are differentiated by two antigens, V and W, and not solely by V antigen alone. The responsibility for resistance may lie with W antigen, or possibly result from the combined action of V and W. While it is evident that V is produced in demonstrable amounts concurrently with the change from sensitivity to resistance whereas W is not demonstrable until some hours after maximum resistance has been achieved under our conditions, this sequence of antigen production may simply reflect the relative ease with which each antigen is detectable. As we have not observed the production of W in the absence of V the decision of the functions of the two antigens must await their separation and purification, and the assessment of their respective biological properties. This work is now in progress.

From studies of immunity Gheltenkoff and Khvorustukina (1940) and Jawetz and Meyer (1943) postulated the presence in virulent strains of a property differentiating them from some natural dissociants, which, apart from complete loss of virulence, appeared to be identical with fully virulent organisms in all other respects. Wats, Wagle and Puduval (1939), Schütze (1939) and Jawetz and Meyer (1943) were unable to demonstrate any antigenic difference between fully virulent and protective avirulent strains by serological tests. The present study has revealed that such strains differ in two antigenic components V and W, one or both of which most probably controls the sole property by which such strains are at present distinguishable *in vitro*, namely ability to develop resistance to phagocytosis in the absence of visible capsulation. Our findings are at variance with those of Englesberg, Chen, Levy, Foster and Meyer (1954) who stated that their studies eliminated the possibility of the existence of an antigen present solely in virulent strains and concluded that virulence was determined by the quantitative relationship between envelope and toxin production.

It has long been known that ability to synthesise the capsule (or " envelope ") antigen, fraction 1, is possessed by all fully virulent strains. Strains lacking this ability are uniformly avirulent. There can be no doubt that fraction 1 antigen is an important determinant of virulence in *P. pestis*. However, many avirulent strains can also synthesise this antigen, which indicates that full virulence requires the presence of at least one other important determinant. This study recognises V (or W) antigens as this additional important factor. Thus fully virulent strains must have the abilities to produce fraction 1 and V (or W) antigens. But at least yet one other determinant of virulence exists since strain EV76, possessing fraction 1, and V and W antigens is of very low virulence. One determinant known to be lacking in EV 76 is that associated with the ability of strains to produce

densely pigmented colonies on defined media containing haemin. The loss of virulence associated with failure to produce pigmented colonies was first observed with the non-pigmented strain M7 derived from the fully virulent pigmented strain MP6 (Burrows, 1955). EV76 is a non-pigmented strain similar to strain M7. The loss of virulence of such strains is the subject of a separate study (Jackson and Burrows, unpublished data).

Some aspects of the sensitisation of resistant virulent organisms with anti-sera have not been resolved. Although high ability to sensitise organisms not visibly capsulated is restricted to anti-sera containing V and W antibodies, sera apparently devoid of these antibodies nevertheless have some activity. Failure to detect these antibodies in such sera may of course be due to insensitivity of our technique. Possibly, however, it indicates the presence of a yet unidentified antibody, having some pro-phagocytic activity and common to all our anti-sera. Preliminary experiments support the latter hypothesis.

Again, it is difficult to explain the observations that anti-sera, produced in rabbits against resistant, virulent, but not visibly capsulated organisms and heavily absorbed with such organisms, have their ability to sensitise these organisms considerably enhanced. The enhancement was only observed in phagocytic systems employing mouse polymorphs. It would appear that such anti-sera contain a component inhibitory to mouse polymorphs, and removable by absorption with the homologous organisms. In the absence of this component and despite some removal of V (or W) antibody these sera become capable of exerting high sensitising ability. Possibly amounts of V (or W) antibody in excess of those required for full sensitisation are inhibitory to mouse polymorphs. Another explanation could be that agglutination by unabsorbed serum reduces phagocytosis by decreasing the frequency of organism-polymorph contacts in the test system. A further alternative could be that organisms, in addition to producing antigen(s) rendering them resistant to phagocytosis, concurrently produce a factor having pro-phagocytic effect. Antibody to this factor would detract from the sensitising value of sera containing V (or W) antibody. Preliminary tests of the above hypotheses have not yielded conclusive results and further work is required to explain this phenomenon.

SUMMARY

Strains of *Pasteurella pestis* possessing the ability to develop resistance to phagocytosis, in the absence of visible capsulation, can produce two antigens (V and W) which other strains, unable to develop such resistance, cannot. All virulent strains examined can elaborate V and W antigens whereas the majority of avirulent strains cannot. Production of V and W antigens among avirulent strains is restricted to those capable of developing resistance to phagocytosis in the absence of visible capsulation. Anti-sera containing demonstrable amounts of V and W antibodies have high ability to render resistant-organisms sensitive to phagocytosis. Anti-sera appropriately absorbed can be used to demonstrate these two antigens by the agar diffusion precipitation technique. V or W, or both, antigens apparently determine the property by which the majority of virulent and avirulent strains can be differentiated *in vitro*, namely the ability of the former to resist phagocytosis in the absence of visible capsulation. For high virulence strains must be capable of producing not only fraction 1 (envelope or capsular)

antigen but also antigen V and/or antigen W. All strains shown to produce antigen V can also produce antigen W. Production of antigen W in the absence of V has not been observed. The avirulent vaccine strain EV76 clearly differs from the avirulent vaccine strain Tjiwidej S in having the potentiality for V and W antigen production and ability to develop resistance to phagocytosis.

We wish to express our gratitude to Dr. K. F. Meyer for generous gifts of purified fraction 1 and its specific anti-serum, and to Dr. G. Girard for supplying an authentic culture of strain EV76 as used for the production of live vaccine. We are also indebted to Dr. D. W. Henderson for his interest and advice, and to Miss Barbara Alkins and Miss Dorothy Burrell for their competent assistance in the laboratory.

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THE USE OF FERMENTATION REACTIONS AND PIGMENT PRODUCTION TO DIFFERENTIATE BETWEEN TYPES OF *PSEUDOMONAS PYOCYANEA* AND OTHER *PSEUDOMONAS* SPECIES, ESPECIALLY *FLUORESCENS*

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PRODUCTION of blue-green pigment and failure to ferment any of the usual coliform test sugars except, occasionally, glucose are generally regarded as characteristic of *Pseudomonas pyocyanea*, the most commonly isolated of the *Pseudomonas* species. In this paper experiments are described showing that under suitable conditions *Ps. pyocyanea* gives a typical fermentation pattern which also differentiates it from *Ps. fluorescens*, not always an easy task. In the course of these fermentation tests red and brown strains of *Ps. pyocyanea* were collected and these fit into Gessard's (1918) scheme as well as the blue-green species; this paper describes results giving excellent agreement between Gessard's colour-typing scheme and the fermentation reactions.

MATERIAL AND METHODS

Seventy-five strains of *Ps. pyocyanea* were isolated in the Bland-Sutton Institute, mainly from routine laboratory examinations of urine and faeces; 7 strains of *Pseudomonas* species were kindly given by the Curator of the National Collection of Bacterial Plant Pathogens at Cambridge, 9 by the Director of Water Examination of the Metropolitan Water Board, 11 strains by Dr. B. W. Lacey of the Westminster Hospital and 26 cultures were obtained from the National Collection of Type Cultures. The total finally realised 99 strains of *pyocyanea*, 5 *fluorescens* and 25 strains including 11 other species and 10 unidentified. All these strains were kept as stabs in broth agar and subcultures were made on broth agar slopes as required.

Fermentation Tests

Sugar media.—Preliminary tests with sugars in 1 per cent peptone water showed that about 80 per cent of the *pyocyanea* strains produced some acid from glucose, giving a pH of 3–6·5. But since it was also observed that the pH of the control cultures was usually as high as 9·5 and that free ammonia could be detected in the air over fluid cultures the possibility that acid from other fermented sugars was being neutralised could not be overlooked.

After testing various synthetic and other media a 1/20 dilution of peptone-free broth in distilled water was found satisfactory, 48-hour cultures grown in oxygen having a final pH of about 7·8.

Two per cent solutions of the chosen sugars were made in the 1/20 diluted infusion broth without added peptone, containing a final concentration of phenol red indicator of 0·002 per cent (added as an aqueous solution since *Ps. pyocyanea* readily produces acid from traces of alcohol). The pH of these solutions was adjusted to 7·6 and they were sterilised by steaming for 30 min. on three successive days. If necessary the pH was adjusted before use by a few drops of freshly boiled 0·1 N-NaOH. Samples of these sugars giving positive fermentation reactions were tested for purity, and especially for the absence of glucose, by paper chromatography.

Tests.—The sugar solutions were distributed in 2 ml. amounts in sterile cotton-wool plugged 3 in. × ½ in. (7·5 × 1·25 cm.) test tubes and inoculated with 2 drops of a suspension of 1 loopful of 18-hr. growth from a broth agar slope in 1 ml. of sterile distilled water. The tubes were placed, with an uninoculated control series, in a capsuleless McIntosh and Fildes jar which was then evacuated to 10 cm. Hg and refilled with oxygen. After 48 hours' incubation (at 37° for *Ps. pyocyanea* and 25° for the other species) the tubes were taken from the jar and allowed to stand on the bench at room temperature for a few hours until the colour of the indicator in the control series returned, when the pH was read using a Lovibond comparator.

Colour Production Tests

Gessard typing.—Gessard (1918) grouped pyocyanin-producing strains of *Pseudomonas* into 16 different types according to the pigment produced in broth peptone water or on peptone agar, as shown in Table I, which is a translation and summary of the Table given in his paper. The "Race" letter A, P, F or S is given for the production in broth of pyocyanin and fluorescin, each alone or neither, as shown in the Table, and the group letter Pe for pyocyanin, M for the brown pigment (melanogen), E for the red pigment (erythrogen) and O for no colour, in peptone water. The glycerol-peptone agar shown in the Table is used by Gessard only for a final pyocyanin check. If no pyocyanin is produced on any of these three media the organism is not regarded by him as *pyocyanea*.

Gessard differentiates clearly between the real red pigment produced by the *erythrogenes* strains and the brownish red seen in old cultures occasionally.

Preparation of Gessard media.—Three media are required. First, broth without peptone; for this ordinary infusion broth without added peptone was used. Gessard describes his second medium as 2 per cent pancreatic peptone solution, but as no more details are given various peptones had to be tried and 2 per cent Difco Bactotryptone was found to be suitable. The third medium is the same 2 per cent peptone water containing 2·5 per cent glycerol and solidified with 1·5 per cent agar. All these media were adjusted to a pH of 7·6 and sterilised by autoclaving. For use 10 ml. amounts were distributed into cotton-wool plugged test tubes and inoculated as were the sugars. The tubes were incubated at 37° for 4 days and then allowed to stand on the bench at room temperature for 6 more days to intensify the colour. The presence of pyocyanin was confirmed by extraction with chloroform at this time.

Potato slices.—These were prepared by the method described in the Medical Research Council's System of Bacteriology (1931). Cylinders were cut with a ¾ in. (1·9 cm.) cork borer from large clean dry potatoes; these cylinders were cut in two obliquely, soaked in water overnight and then in 6 per cent glycerol saline for one hour. Each section was then placed in a screw-cap tube, resting on a one-inch wad of absorbent cotton-wool saturated with the 6 per cent glycerol saline solution, and the tubes were sterilised by steaming. Slices which turned black were rejected. A fairly heavy suspension was used as inoculum, a few drops being allowed to run down the centre of a slope; this resulted in confluent growth down the length of the slope having a width of approximately 0·5 cm. The rubber lining of the caps was removed when the tubes were inoculated and the tubes were incubated for 4 days at 37° and were then kept on the bench at room temperature for another 6 days, by which time the cotton-wool was often intensely coloured also.

RESULTS

The results of the sugar fermentation tests are shown in Table II. All of the 99 strains of *Ps. pyocyanea* tested fermented D-arabinose and 89 strains fermented in addition L-arabinose, D-glucose, D-xylose and D-galactose; 20 of these latter strains fermented trehalose. The pH of fermented tubes was less than 6·8 and of other tubes 7·6 rising to more than 8·4 after 24 hours on the bench. Growth appeared to continue in the unfermented tubes on the bench but not in the acid tubes. The positive reactions are listed in Table II but the 99 strains were also tested against, and failed to ferment, lactose, salicin, dulcitol, raffinose, sucrose, mannitol, maltose and dextrin.

TABLE I.—*Gessard's Classification of* Ps. pyocyanea *by Colour Production in Broth, Peptone Water and Peptone Agar Cultures.*

Colour produced after 10 days in: Broth.	"Race" letter.	Peptone water.	"Race" letter.	Glycerol-peptone agar.	"Race" letter.	Gessard type.
Blue (pyocyanin)	P	Blue	Pe	..	..	PeP
Ditto	P	Brown	M	..	..	MP
,,	P	Red	E	..	..	EP
,,	P	*Nil*	O	..	..	OP
Fluorescent blue-green (pyocyanin + fluorescin)	A	Blue	Pe	..	..	PeA
Ditto	A	Red	E	..	..	EA
,,	A	Brown	M	..	..	MA
,,	A	*Nil*	O	..	..	OA
Fluorescent yellow-green (fluorescin)	F	Blue	Pe	..	..	PEF
Ditto	F	Brown (− pyocyanin)	..	Blue	M	MF
,,	F	Brown (+ pyocyanin)	M	..	..	MF
,,	F	Red (− pyocyanin)	..	Blue	E	EF
,,	F	Red (+ pyocyanin)	E	..	..	EF
,,	F	*Nil*	..	Blue	O	OF
Nil	S	Blue	Pe	..	..	PeS
,,	S	Brown (− pyocyanin)	..	Blue	M	MS
,,	S	Brown (+ pyocyanin)	M	..	..	MS
,,	S	Red (− pyocyanin)	..	Blue	E	ES
,,	S	Red (+ pyocyanin)	E	..	..	ES
,,	S	*Nil*	..	Blue	O	OS
,,	S	*Nil*	..	*Nil*	..	Not *pyocyanea*

TABLE II.—*Fermentation Reactions and Colour Production on Potato Slices of 99 Strains of* Ps. pyocyanea *Divided into 7 Gessard Types by their Colour Production in Broth, Peptone Water and Glycerol-peptone Agar*

Gessard type.	Number of strains.	Number of strains producing acid from: D-arabinose.	L-arabinose.	D-glucose.	D-galactose.	D-xylose.	Trehalose.	Potato slices. Colour of growth.	Colour of medium.
PeP	8	+	−	−	−	−	−	Nut-brown	Blue-green
PeA	35	+	+	+	+	+	10	,,	,,
	20	+	+	+	+	+	3	Blue-green	,,
OA	6	+	+	+	+	+	−	,,	,,
	6	+	+	+	+	+	1	Nut-brown	,,
OF	13	+	+	+	+	+	5	Dry-pink	*Nil*
MA	3	+	+	+	+	+	1	Nigger-brown	Nigger-brown
EA	4	+	+	+	+	+	−	Red	Red
EF	4	+	+	+	+	+	−	Red	Red

Reactions with the sugars are given thus: + = all strains positive; − = all strains negative; numeral = number of strains positive.

It will also be seen in Table II that there are 8 strains which ferment only D-arabinose, and that these strains are different from the other 89 in failing to produce fluorescin in broth cultures, and are therefore in Gessard type PeP, producing only pyocyanin (Table I). The 20 per cent of strains which ferment trehalose, on the other hand, are not confined to any one Gessard type but fairly evenly distributed although it is not possible to judge in the groups with a few strains only.

The 7 Gessard types found amongst the 99 strains are also shown in Table II and include red and brown strains, but over half (55 strains) are of type PeA. Cultures of this type are the usual blue-green producing strains, both broth and peptone water cultures forming pyocyanin and broth cultures fluorescin also, the precise tint of the broth culture depending on the relative amounts of each pigment present. Many so-called "*fluorescens*" cultures reveal appreciable quantities of pyocyanin when shaken with chloroform; there is however some tendency for the cultures with more fluorescin to be in the half of the group which shows brown growth on potato rather than green (Table II) although all the strains of this type colour the potato blue-green; the 12 strains of the related type OA (Tables I and II) are divided in the same way on potato, while producing a blue-green background, and it seems likely that this colour, a light nut-brown, quite different from the nigger brown of the M types, is due to the presence of fluorescin, since the true *fluorescens* cultures show the same colour growth but do not colour the potato at all.

The three MA (dark brown) strains, as shown in Table II, ferment the same sugars as the blue-green types, and produce pyocyanin and fluorescin in broth, but in peptone water they produce much dark brown pigment in addition. This is also strikingly shown on the potato slopes where the brown gradually spreads out over the more rapidly produced green. The red pigment produced by the erythrogenic strains (EA and EF) in peptone water is also very marked on the potato slices (Table II), even the cotton-wool becoming a deep rose-red in a few days. This red pigment differs from the browner red colouration sometimes seen in old *pyocyanea* cultures, both in its tone and in the effect upon it of acid and alkali. The effects of strong hydrochloric acid and ammonium hydroxide on the various pigments are summarised in Table III, and it will be seen that while the red and brown pigments are the same colour at pH 3 and pH 11, fluorescin is decolourised by acid, the colour being restored by ammonia, the acid pyocyanin is red brown and can be turned back to blue by adding ammonia.

TABLE III.—*Effects on the Pigments Produced by* Ps. pyocyanea *in Broth or Peptone Water of Changing the pH from High to Low or* vice versa

Original colour of broth or peptone water culture (at pH 9·0).	Effect on colour of culture of adding sufficient acid or alkali to change the pH to	
	3·0.	11·0.
Fluorescent yellow-green (fluorescin)	Colourless	Fluorescent yellow-green
Blue (pyocyanin)	Red-brown	Blue
Rose-red	Rose-red (unchanged)	Rose-red (unchanged)
Nigger-brown	Nigger-brown (unchanged)	Nigger-brown (unchanged)

Acidification was with 10N-HCl, alkalisation with 5 N-NH_4OH.

The last remaining type shown in Table II is type OF. These strains are very different from the other in that they are only pale green in broth, and colourless in peptone water, but all produce pyocyanin in peptone agar. As shown in Table II their growth on potato is a bright salmon pink and has a dry appearance, while the potato is uncoloured. Were it not for the fermentation, the pyocyanin production on peptone agar and the different growth on potato these strains might have been thought to be *fluorescens*.

TABLE IV.—*Fermentation Reactions of* 30 *Strains Belonging to* 13 Pseudomonas *Species other than* pyocyanea

Pseudomonas species.	Number of strains.	D-arabinose.	L-arabinose.	D-glucose.	D-galactose.	D-xylose.	Trehalose.	Sucrose.	Mannite.	Maltose.
Fluorescens	5	−	+	+	+	+	−	−	−	−
Ovalis	2	−	+	+	+	+	−	−	−	−
Mildenbergii	1	−	+	−	+	+	−	−	−	−
Ichthyosmia	2	−	+	+	+	1	+	+	+	+
Hydrophila	2	−	+	+	1	1	1	1	1	1
Indigoferra	1	−	+	+	+	−	+	+	+	+
Crusiviae	1	−	−	+	−	−	−	−	−	−
Oleovorans	1	−	−	+	−	−	−	−	−	−
Phascolicola	1	−	+	+	+	+	−	+	−	−
Syringae	2	−	+	+	+	+	−	+	+	−
Viridiflava	1	−	+	+	+	+	+	−	+	−
Viridilivida	1	−	+	+	+	+	+	−	+	−
Unidentified	5	−	+	+	+	+	+	+	+	−
	5	−	+	+	+	+	−	−	−	−

Reactions with the sugars are given thus: + = all strains positive; − = all strains negative; numeral = number of strains positive.

The difference between these OF strains of *Ps. pyocyanea* and true *Ps. fluorescens* is shown in Table IV, which shows that the *fluorescens* strains ferment D-glucose, D-xylose, D-galactose and L-arabinose but not D-arabinose. In addition they form no pyocyanin and do not colour potato slopes, on which their growth is a light nut-brown colour. As shown in Table IV strains of other non-pyocyanin producing species of *Pseudomonas* were tested for fermentation and although various sugars were attacked none of the strains formed acid from D-arabinose. None of the species shown in Table IV showed any colour in peptone water or on potato slices, and only a pale yellow green colour insoluble in chloroform in broth.

All of the *pyocyanea* strains liquified gelatin within 7 days, the apparent form of the liquifaction progressing with time from crateriform through infundibuliform and saccate to stratiform and complete liquifaction. Litmus milk showed reduction and digestion within 7 days. None of the strains listed in Table IV except the two *hydrophilus* attacked gelatin during 52 days, and only one of the *ovalis* strains, which showed slight traces of acid, attacked litmus milk during 42 days.

DISCUSSION

The great difficulty with *Ps. pyocyanea* has always been to decide whether or not the colourless and yellow-green types were *pyocyanea* or *fluorescens*. From the experiments described above, it is clear that there are two methods of distinguishing between these species, both giving the same answer. These methods

are the fermentation of D-arabinose in peptone-free medium, and Gessard's colour-typing scheme, which appears to have been neglected unaccountably. By the combination of these two methods as shown in Tables II and IV it is clear that all *pyocyanea* strains produce pyocyanin in one or more of Gessard's media and ferment D-arabinose, while *fluorescens* (and the other species) do not. The perfect agreement between the fermentation and Gessard typing is very striking.

The *pyocyanea* strains having been identified can then be further grouped using Gessard's method summarised in Table I, and this result also can be verified by another method since as shown in Table II the basic colours can also be observed on potato slices, even the colourless types giving a pink growth typical of the group.

There does appear from the results given in Tables II and IV to be a strong correlation between the fermentation of D-arabinose and production of pyocyanin ; this is emphasised by the fact that the strains of type PeP, producing only pyocyanin, ferment only D-arabinose. This is the only significant difference in fermentation reactions within the *pyocyanea* group, since as shown in Table II all the types ferment the same four sugars and trehalose fermentation is distributed over the types fairly equally.

There are not a sufficient number of the other species to be of significance apart from the fact that none ferments D-arabinose.

SUMMARY

The fermentation of sugars by *Pseudomonas* cultures is masked in the peptone water medium usually employed by the production of free ammonia and a consequently high pH. This can be avoided by using dilute peptone-free broth as a base.

All strains of *Ps. pyocyanea* tested (total 99) ferment D-arabinose and 90 per cent ferment also L-arabinose, D-glucose, D-galactose and D-xylose ; 25 strains of other *Pseudomonas* species including 5 *fluorescens* while fermenting other sugars fail to ferment D-arabinose.

The strains of *Ps. pyocyanea* have been grouped into 7 of Gessard's 16 types, all of them producing pyocyanin in at least one of his media. Similar colour reactions have been obtained on potato slices.

It has been possible to differentiate between *fluorescens* and *pyocyanea* cultures since all of the latter produce pyocyanin in Gessard media and ferment D-arabinose, neither of which conditions is fulfilled by *fluorescens*.

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THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTIBACTERIAL ACTIVITY. PART VIII. 2-MERCAPTOPYRIDINE-N-OXIDE, AND SOME GENERAL OBSERVATIONS ON METAL-BINDING AGENTS

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8-HYDROXYQUINOLINE (oxine) (I) and its derivatives are powerful bactericides, and the antibacterial action has been traced to their ability to chelate, *i.e.*, to form cyclic complexes with the ions of heavy metals (Albert, Rubbo, Goldacre and Balfour, 1947). Further, these substances are innocuous if traces of iron are removed from the testing medium (Rubbo, Albert and Gibson, 1950). Evidence points to the 1 : 1 oxine : iron complex as the true toxic agent, and to the interior of the bacterial cell as its site of action (Albert, Gibson and Rubbo, 1953). This work led to the examination of hydroxy-derivatives of quinoxaline, quinazoline and similar substances all derived from the 8-hydroxyquinoline series by the insertion of further ring-nitrogen atoms. It was concluded that a high oil/water partition coefficient (*i.e.*, in favour of lipids) is necessary (Albert, Hampton, Selbie and Simon, 1954). As high coefficients are known to assist penetration into the cell, this was taken as supporting the hypothesis that action takes place within the cell.

It has often been postulated, but not upheld, that some chelating agents act against bacteria by sequestering essential heavy metals such as iron. For example, it has been suggested that the 2-substituted pyridine-N-oxides act by this mechanism (Lott and Shaw, 1949). As such a starvation mechanism is entirely different from the one which has been established for oxine in this series of papers, an investigation of these pyridine-N-oxides, *e.g.*, (III), was undertaken and is reported here. It is found that the mode of action is apparently identical with that of oxine although the substances are chemically so different. Further work is reported on the broader issue of what properties a metal-binding agent must possess to be antibacterial.

EXPERIMENTAL

Microbiology

The greatest dilution preventing visible growth was used as the criterion in the bacteriostatic tests. The strains of bacteria used were intermediate (in sensitivity to oxine) between various strains used in earlier papers in this series. Hence oxine was included in the tests, as a control, so that the antibacterial action of the new substances could be compared with it as a standard.

The bacteriostatic tests consisted of inoculating two series of twofold dilutions (beginning at M/200 and M/2000) of the substance in broth (pH 7·3) and inspecting the solutions for growth after 48 hours' incubation at 37°. Each test was made in duplicate. The bactericidal tests reported in Table IV were carried out by the technique of Miles and Misra (1938).

The mould *Penicillium spinulosum* was grown in 2 per cent malt extract (Allen and Hanbury). To 100 ml. of the solution 20 ml. of McIlvaine's citric acid disodium phosphate buffer of the appropriate pH was added. The pH was adjusted to the desired figure (by adding additional citric acid or disodium phosphate) using a glass electrode, the medium bottled in appropriate volumes and sterilised by steaming. The inoculum in all cases was one drop of a spore suspension obtained from growth of the mould on solid medium.

Chemistry

The preparation, ionisation constants, stability constants and partition coefficients of the various substances used are given in Table I.

Ionisation constants were determined by titration of 0·0005 M of the dried specimen in air-free water under nitrogen, using a Cambridge pH set with glass and calomel electrodes (standardised to pH 4·00 with 0·05 M potassium hydrogen phthalate, and to 9·20 with 0·05 M borax, at 20°). Results were acceptable only when agreement on re-standardisation with these buffers after titration lay within ± 0·01 unit. Nine-tenths of an equivalent of 0·1 N hydrochloric acid, or potassium hydroxide (carbon-dioxide free), was added in nine equal portions. The nine pK_a values for each pH reading were calculated, when acid was the titrant, from the formula :

$$pK_a = pH - \log([B] + [H^+] / [BH^+] - [H^+])$$

where $[BH^+]$ and [B] are the concentration of the molecule, protonated and non-protonated respectively, neglecting hydrolysis corrections which are taken care of by the remainder of the formula. When alkali was the titrant, the following formula was used :

$$pK_a = pH + \log([AH] + [OH^-]/[A^-] - [OH^-])$$

The nine pK values were converted into antilogarithms before averaging, then reconverted to logarithms. The values were rejected if the spread exceeded ± 0·06.

Stability constants were determined by adding an equivalent of 0·1 N potassium hydroxide (carbonate-free) in ten equal portions to aqueous solutions (0·001–0·0025 M) which contained the substance and one equivalent of the metallic ion (*e.g.*, cobalt nitrate). When titrating substances No. 8 and 9 in the presence of copper, prior addition of hydrochloric acid was necessary to reveal the lower values of $\bar{n}$ (defined as the average number of molecules of chelating substance combined with one atom of metal). The titrations were carried out in boiled-out water, under nitrogen. Calculations of the stability constants were made as described by Albert (1950, 1952). The symbol K′ refers to the equilibrium betwen the substance and the 1 : 1-complex, *e.g.*, (IV) ; whereas K″ refers to the equilibrium between the 1 : 1- and the 2 : 1- complex, *e.g.*, (V).

Partition coefficients, between purified oleyl alcohol and 0·05 M buffer, were carried out (under nitrogen for No. 2) as described by Albert and Hampton (1954).

The substances were prepared as in Table I, or as follows. 2-(2′-Hydroxyphenyl) pyridine (IX), prepared according to Geissman (1946), proved to be not an oil, as reported, but a solid of m.p. 53°. (Found : C, 76·7 ; H, 5·3 ; N, 7·9. Calculated for $C_{11}H_9ON$, C, 77·1; H, 5·3 ; N, 8·2 per cent). 3-Butyryl-2-hydroxy-6-propyl-γ-pyrone, prepared according to Deshapande (1932), and fractionated melted 9° higher (*viz.* 25°) than in the literature. (Found : C, 64·0 ; H, 7·0. Calculated for $C_{12}H_{16}O_4$, C, 64·3 ; H, 7·2 per cent.) *o*-Hydroxyacetophenone (XIV) was a gift from Professor A. Birch. 1-Hydroxy-2-acetonaphthone was prepared according to Witt and Braun (1914), m.p. 98°. Hydrazine-N : N-diacetic acid (XI) was made according to Bailey and Read (1914). (Found : C, 32·3 ; H, 4·9 ; N, 18·9. Calculated for $C_4H_8O_4N_2$, C, 32·4 ; H, 5·4 ; N, 18·9 per cent). 4-Butyl-2-aminophenol (XIII) was prepared from phenyl butyrate by rearrangement, reduction (Rice and Harden, 1936), nitration (Close, Tiffany and Spielman, 1949), and final reduction (Baranger, 1931).

RESULTS

The results of the bacteriostatic testing of five heterocyclic N-oxides (No. 2–6) bearing mercapto- or hydroxy- groups in the 2-position are given in Table II. Oxine is included as a standard, for comparison. Logarithmic stability constants

TABLE I.—*Preparation and Physical Properties of Test-substances*

No.	Substance.	Preparation.	Ionisation (pKa, 20°). Basic.	Ionisation (pKa, 20°). Acidic.	Stability constants (logs ; 20°). Copper. K′.	Copper. K″.	Nickel. K′.	Nickel. K″.	Zinc. K′.	Zinc. K″.	Cobalt. K′.	Cobalt. K″.	Ferrous. K′.	Ferrous. K″.	Partition coefficients (oleyl alcohol/water) 20°. pH 7·3.	pH 3·0.
1	8-Hydroxyquinoline (oxine) for comparison	a	5·13	9·89	12·2[l]	n	9·9	n	8·6	p	9·1	p	8·0	p	67	—
2	2 - Mercaptopyridine - N - oxide (III)	b	<0·5	4·57	m	m	m	m	5·9	5·4	5·5	4·5	4·7	m	1·07	—
3	2 - Mercapto - 4 - *n* - propyl - pyridine-N-oxide	c	j	j	m	m	m	m	m	m	m	m	m	m	q	—
4	2 - Hydroxypyridine - N - oxide	d	−0·62[k]	5·97	7·0	6·2	5·7	4·4	5·5	4·3	5·3	4·3	m	m	0·03	—
5	2 - Hydroxyquinoline - N - oxide (VI)	e	<1	6·88	m	m	5·5	m	5·4	m	5·1	m	m	m	3·18	—
6	2-Hydroxy-4-methyl-5 : 6-benzoquinoline - N - oxide (VIII)	c	j	j	m	m	m	m	m	m	m	m	m	m	r	—
7	2-(2′-Hydroxyphenyl) pyridine (IX)	f	4·20	10·63	m	m	6·1	m	m	m	—	—	—	—	—	—
8	2-(2′-Hydroxyphenyl) imidazoline	g	6·63 <1	12·58	11·7	m	8·1	m	7·9	m	7·5	6·5	m	m	0·36	—
9	2 - (2′ - Hydroxynaphthyl - 3′-) imidazoline (X)	c	7·01	10·85	11·3	10·6	—	—	—	—	—	—	—	—	7·47	—
10	Hydrazine - N : N - diacetic acid (XI)	f	7·32	2·40 3·12	m	m	7·8	5·2	6·7	m	—	—	—	—	0·05[s]	—
11	Dehydracetic acid (3 - acetyl-2-hydroxy-6-methyl-γ-pyrone) (XII)	a	<1	5·53	5·6	4·3	4·1	m	m	m	m	m	m	m	0·3	8·9
12	3-Propionyl-2-hydroxy-6-ethyl-γ-pyrone	h	<1	5·38	5·3	m	3·9	m	m	m	m	m	m	m	2·3	156
13	3 - Butyryl - 2 - hydroxy - 6 - propyl-γ-pyrone	f	<1	5·47	m	m	m	m	m	m	m	m	m	m	10·5	18,800

— = Not attempted.
a = Commercial, recrystallised to constant m. pt.
b = Shaw *et al.* (1950).
c = Rees (1956).
d = Lott and Shaw (1949).
e = Newbold and Spring (1948).
f = See text, under "Experimental."
g = Johnston and Freiser (1954) [but higher m.p., *viz.* 203–4°].
h = Deshapande (1932).
j = Not determinable because of poor solubility.
k = Determined spectrometrically.

l = All oxine figures from Albert (1953), except zinc which is from Näsänen (1952).
m = Sparing solubility interfered with attempted determination.
n = Not exactly determinable because of sparing solubility, but estimated to be *two* units less than log K′.
p = As n, but *one* unit less.
q = as m, but estimated to be > 30.
r = As m, but estimated to be > 10.
s = The oleyl alcohol layer was diluted with ethanol and titrated with alkali.

for the binding of zinc are included as a sample of the metal-combining powers of each substance (other stability constants are available from Table I). It is evident that all the N-oxides have similar stability constants, which are about a thousand times less than that of oxine [*e.g.*, or No. 1 and 4, antilog (8·6–5·5) = 1300]. One substance (2-mercaptopyridine-N-oxide) has an antibacterial action much greater than that of oxine. The antibacterial action of the 2-hydroxy-derivatives is far inferior to that of the 2-mercapto-derivatives. In the 2-hydroxy- series, the antibacterial action is seen to increase as the partition coefficient rises (*i.e.*, as the molecule is modified so as to favour dissolution in lipids at the expense of water).

Substances 2–6 differ from oxine in that the metal is located between two oxygen atoms, or between an oxygen and a sulphur atom. Substances 7–10, 14 and 15 were synthesised to provide the same type of chelation as in oxine, the metal being held in a five or six-membered ring between an oxygen and a nitrogen atom. None of these substances approaches oxine in antibacterial power, but in the pairs 8–9 and 14–15 an increase in activity with increasing lipophilic nature can be seen.

TABLE II.—*Bacteriostatic Action of Test Substances*

Highest M dilutions preventing visible growth in 48 hr. at 37° (medium : broth, pH 7·3.

No.	Substance.	*Str. pyogenes.*	*Staph. aureus.*	*Bact. coli.*	Stability constant (log K′) zinc.	Partition coefficient pH 7·3 (see Table I).
1	Oxine (for comparison)	40,000	40,000	<5,000	8·6	67
2	2-Mercaptopyridine-N-oxide (III)	160,000	160,000	10,000	5·9	1·07
3	2-Mercapto-4-*n*-propylpyridine-N-oxide	64,000	32,000	3,000	5·9†	>30†
4	2-Hydroxypyridine-N-oxide	100	200	100	5·5	0·03
5	2-Hydroxyquinoline-N-oxide (VI)	1,000	1,000	1,000	5·4	3·18
6	2-Hydroxy-4-methyl-5:6-benzoquinoline-N-oxide (VIII)	4,000	4,000	2,000	5·5†	>10†
7	2-(2′-Hydroxyphenyl)-pyridine (IX)	—	200	—	6†	—
8	2-(2′-Hydroxyphenyl)-imidazoline	800	800	<200	7·9	0·36
9	2-(2′-Hydroxynaphthyl-3′)-imidazoline (X)	2,000	2,000	<1,000	7·9†	7·47
10	Hydrazine-N:N′-diacetic acid (XI)	100	200	100	6·7	0·05
14	2-Aminophenol	3,200	1,600	200	4‡	0·40
15	4-Butyl-2-aminophenol (XIII)	8,000	4,000	<4,000*	4‡	7·06

— = Not attempted.
* = M/4000 is strongest solution obtainable in broth, and this was inactive.
† = Estimated from values of nearest analogues.
‡ = Estimated from Charles and Freiser (1952) who worked in dioxane.

Table III relates the antifungal action of dehydracetic acid, and its homologues, to the pH at which the test is carried out, and hence to the percentage of non-ionised substance present. At a pH suitable for testing bacteria, these

substances are completely ionised and it can be seen from Table III that they are not antibacterial.

TABLE III.—*Fungistatic and Bacteriostatic Action in the Dehydracetic Acid Series*

No.	Substance.	*Penicillium spinulosum.** Highest dilution preventing visible growth. pH 4.	pH 8 (buffered).	Percentage not ionised. pH 4.	pH 8.	*Str. pyogenes,† Staph. aureus* and *Bact. coli.* Highest concentrations obtainable. These did not prevent growth.	Percentage not ionised at pH 7·5.
11	Dehydracetic acid (3-acetyl-2-hydroxy-6-methyl-γ-pyrone (XII)	4,000	200	97	0·32	200	0·99
12	3-Propionyl-2-hydroxy-6-ethyl-γ-pyrone	16,000	<1,000	96	0·25	1,000	0·79
13	3-Butyryl-2-hydroxy-6-propyl-γ-pyrone	32,000	2,000	97	0·32	2,000	0·99

* Heavy inoculum of spores, incubated for 7 days in 2 per cent malt extract at 30°. † Each in broth at pH 7·5 and 30°.

As 2-mercaptopyridine-N-oxide (No. 2) has high antibacterial activity, it was subjected to tests which had previously done much to reveal the mode of action of oxine (Albert *et al.*, 1953). Table IV shows that, like oxine, this substance is bactericidal only in the presence of iron, this activity being prevented by traces of cobalt, but not of nickel. Increasing the ratio of substance to iron abolishes the activity, as with oxine.

TABLE IV.—*The Effect of Traces of Metallic Cations on Bactericidal Action in Glass-distilled Water (pH 6·0–6·8) at 20°*

Organism : *Staph. aureus.*

Tube. No.	2-Mercaptopyridine-N-oxide 1/M.	Ferric sulphate 1/M.	Growth after exposure (hr.). 0.	2.
1	20,000	*Nil*	+++*	+++
2	20,000	20,000	−†	−
3	40,000	*Nil*	+++	+++
4	40,000	40,000	+++	−
5	80,000	*Nil*	+++	+++
6	80,000	80,000	+++	−
7	160,000	*Nil*	+++	+++
8	160,000	160,000	+++	+
9	80,000	80,000	+++	−
10	8,000	80,000	+++	+++
11	800	80,000	+++	+++
12	40,000	40,000 + 40,000 Co^{++}	+++	+++
13	40,000	40,000 + 40,000 Ni^{++}	+++	−

* +++ signifies discrete colonies, but uncountable ; + signifies 10 to 50 colonies ; −no growth.
† Rapid bactericidal action before count could be made.

Finally, the partition coefficients of oxine and some of its derivatives were determined to provide necessary data for comparison. These are reported in Table V, together with the bacteriostatic figures for these substances.

TABLE V.—*Correlation of Bacteriostatic Activity and Partition Coefficient in the Oxine Series*

Highest dilution (expressed as 1/M) preventing visible growth in 48 hr. at 37° (medium : broth, pH 7·3).

No.	8-Hydroxyquinoline.	*Str. pyogenes.**	*Staph. aureus.**	Partition coefficient oleyl alcohol/water 20° : pH 7·3.
1	(Unsubstituted, *i.e.*, oxine)	100,000	100,000	67
17	5 : 7-Dichloro-	100,000	100,000	294
18	5 : 6-Benzo-	200,000	26,000	234
19	-5-Carboxylic acid	3,200	<800	3·90

* Bacteriostatic values from Albert *et al.* (1947), the strains being slightly more sensitive than in the present work.

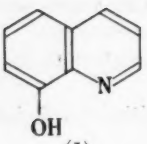
DISCUSSION

2-*Mercaptopyridine-N-oxide* (III)

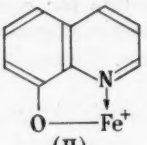
High antibacterial powers were claimed for this substance at the time of its discovery (Shaw, Bernstein, Losee and Lott, 1950; Pansy, Stander, Koerber and Donovick, 1953). However, it has never been subjected to comparative testing alongside oxine (8-hydroxyquinoline) or other potent antibacterials. This has now been done and it is evident from Table II that this substance is four times as active as oxine against the Gram-positive species. It also shows significant activity against the Gram-negative organism, but of a lower order.

Because 2-mercaptopyridine-N-oxide (also known as N-hydroxy-2-pyridine-thione) is so potent, its mode of action was investigated. As recorded in Table I, it forms complexes with the cations of heavy metals. Yet it binds these metals differently from oxine, because it holds the metal between oxygen and sulphur, see (IV), whereas oxine holds the metal between oxygen and nitrogen, see (II). Nevertheless a common mode of action is not excluded, and hence the mercapto-compound was submitted to four tests which are characteristic of oxine, and which have shed much light on the mode of action of oxine. These tests are reported in Table IV.

It is characteristic of oxine that (*a*) it is rapidly bactericidal, (*b*) it is bactericidal only if traces of iron are present, (*c*) such traces of iron do not activate oxine if traces of cobalt (but not nickel) are added, and (*d*) traces of iron do not activate in the presence of an excess of oxine (Albert *et al.*, 1953). It is evident from Table IV that 2-mercaptopyridine-N-oxide follows the same pattern. Tube 2 reveals its rapid bactericidal action, and a comparison of tubes 1 and 2, 3 and 4, 5 and 6 shows that it is inactive in the absence of small amounts of iron. The de-activating effect of cobalt on an iron-activated mixture is seen by comparing tubes 12 and 4 (tube 13 shows that, as with oxine, nickel has no comparable effect). Finally the quenching effect of an excess of 2-mercaptopyridine-N-oxide is seen in tubes 9–11.



(I)



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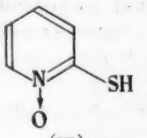

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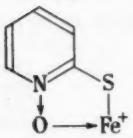

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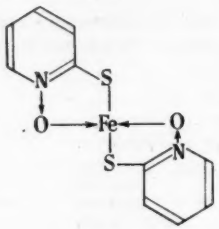

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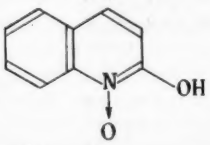

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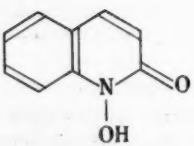

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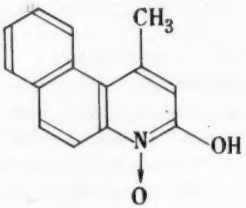

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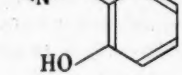

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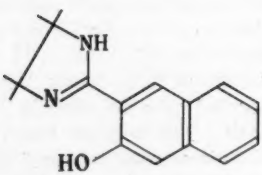
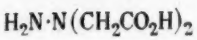
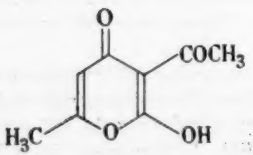


(X)



(XI)



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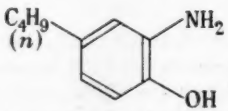
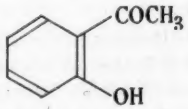


(XIII)



(XIV)

[The de-activating effect of cobalt has been demonstrated in two other types of metal-binding antibacterials : 5-bromosalicylal-thiosemicarbazone and 1-nitroso-2-naphthol (Schraufstätter, 1950).]

Thus it must be concluded that, like oxine, 2-mercaptopyridine-N-oxide is not of itself toxic, but that it forms a toxic 1 : 1-complex (IV). From tubes 9–11 of Table IV, it is evident that the 2 : 1-complex (V) is not toxic, just as in the case of oxine.

The hypothesis has been put forward that the oxine-metal complex acts inside the cell (Albert *et al.*, 1953). This concept received strong support from further work in which a series of analogues of oxine were tested. In these substances the high oil-water partition coefficient of oxine was lowered by inserting an extra ring-nitrogen atom in all possible positions (Albert *et al.*, 1954). Because of the low coefficients, such substances are less likely to enter the cell. As the partition-coefficients fell, the antibacterial activity also fell ; and when (still retaining the extra nitrogen atom) the partition coefficient was raised by inserting a small alkyl-group, the antibacterial action also increased, exactly as the hypothesis demands. At the time, no investigation was made to see if the partition coefficient of oxine was optimal, or if raising the coefficient would improve the action. This has now been investigated, and it would seem from Table V that a fourfold increase in the coefficient confers no extra benefit (compare No. 1, 17 and 18). Likewise, the carboxylic acid derivative of oxine (No. 19) which has only feeble antibacterial properties, is now seen to have a low coefficient, as was predicted earlier (Albert *et al.*, 1947). It must be pointed out that these correlations of partition coefficients with antibacterial activity of the substances in Table V are valid only if the stability constants do not vary appreciably. This may safely be assumed for substances 1, 18 and 19 at least, because annelation has little effect (compare No. 4 and 5 in Table I), and oxine-5-sulphonic acid has almost the same stability constants as oxine (Albert, 1953).

This work on the derivatives of oxine (particularly that reported in 1954) suggests that other series of antibacterials which act in conjunction with metals would also be benefited by increasing the partition coefficient, at least until an optimal value was reached. It is not simple to subject 2-mercaptopyridine-N-oxide to this hypothesis as it is somewhat prone to destruction by oxidation, and this tendency is greatly magnified by the introduction of alkyl-groups necessary to raise the partition coefficient. Indeed, another analogue intended for the present series (2-mercapto-4-methyl-5 : 6-benzoquinoline-N-oxide) was so readily oxidised by air (Rees, 1956) that difficulty was found in determining the physical constants. Thus, it is not necessarily contrary to the hypothesis that No. 3 (Table II) seems to be less antibacterial than No. 2.

Fortunately the corresponding hydroxy-compounds (No. 4, 5 and 6) have no tendency to oxidise. These three substances demonstrate the postulated increase in antibacterial action with rising partition coefficient, a satisfactory comparison because the affinity for metals (as shown by the stability constants) is of the same order. Further small series showing this effect are No. 8 and 9, also No. 11, 12 and 13 in Table III and apparently No. 14 and 15 (Table II).

However, it is evident that such a rule can be applied only within a series of related substances. For example, 8-hydroxyquinoline-5-carboxylic acid is almost inactive because its partition-coefficient is 3·9, a figure that is too low for activity in the oxine series (Table V ; also see Albert *et al.*, 1954 for much other relevant

data). Yet, in another series, the lower figure of 1·07 suffices for an outstandingly active substance, 2-mercaptopyridine-N-oxide (No. 2).

The stability constants of this mercapto-compound also fall below those of oxine. Obviously, for a substance to be able to act through forming metal complexes the stability constants must exceed certain critical values. It is now suggested that these critical values are the stability constants of the common amino-acids, in so far as the antibacterial will be in competition with these (free and combined) in the medium, and inside the cell. The figures for glycine (these are typical of those of amino-acids in general, excepting histidine and cysteine, which have higher avidity) are :

Logarithmic stability constants for glycine

Copper.		Nickel.		Zinc.		Cobalt.		Iron (ferrous).	
K′	K″	K′	K″	K′	K″	K′	K″	K′	K″
8·5	6·9	6·1	5·0	5·3	4·1	5·1	3·9	4·3	3·7

2-Mercaptopyridine-N-oxide has K′ values not much above this supposed critical level. For example its affinity for zinc is :

$$\text{antilog } (5{\cdot}9–5{\cdot}3) = 4$$

that is to say only four times greater than glycine, comparing the 1 : 1 complexes, *e.g.*, (IV). But an 80-fold difference in favour of the mercapto-compound is seen if the second stability constant (K″) is also used, and thus the 2 : 1-complex, *e.g.*, (V), is taken into consideration. Actually, the 2 : 1 complex of oxine, being liposoluble like oxine itself, has been suggested to be the form in which oxine penetrates the cell, because the 1 : 1-complex is non-liposoluble, and presumably non-penetrating. In the presence of excess oxine the 2 : 1-complex is held as such in the cell and so the cell is unharmed, but in the absence of excess oxine, the 2 : 1-complex is broken down to the toxic 1 : 1-complex or further, according to this hypothesis (Albert *et al.*, 1953). It was shown above that the mercapto-compound has the same mode of action as oxine, and hence the constants of the 2 : 1-complex should be significant for this substance also. Analogues of oxine with reduced stability constants seem to be less active than oxine (Albert *et al.*, 1954), so no virtues can be claimed for the mercapto-compound's nearness to the supposed critical figures.

To sum up : 2-mercaptopyridine-N-oxide has lower stability constants, and lower partition coefficients than oxine, but is nevertheless more active biologically. This points to the likelihood of a third factor playing a part. This factor can hinge on a very small change in the molecular structure as is seen by comparing No. 2 with No. 4 and 5 (Table II). These substances have stability constants and partition coefficients of the same order, yet No. 4 and 5 are much poorer as antibacterials. The significant difference between these substances is whether they have sulphur or oxygen in the 2-position, the substances with oxygen being chemically stable, whereas those with sulphur are prone to oxidative destruction. As was mentioned above, the metal-binding antibacterials are thought to enter the bacterial cell as the 2 : 1-complex with iron, but to act only after this has been broken down. Such a breakdown would be particularly easy in the case of mercapto-compounds

which readily form disulphides, a class of substances which cannot bind metals. It is therefore suggested that the third factor is this ease of chemical change. Thus a limited proneness to chemical decomposition may be an advantage.

General observations on metal-binding agents

Given that these three factors are significant in imparting antibacterial properties to a metal-binding substance, the question arises : How can knowledge of these factors lead to the discovery of new types of metal-binding antibacterials?

The partition-coefficient is the factor most easily brought under control. It can be raised considerably by inserting a small side-chain of perhaps not more than three carbon atoms, as in the aza-oxines (Albert *et al.*, 1954). But this rule does not apply if the substance is ionised at the pH of the biological tests (information which is readily obtained from the ionisation constant).

By their nature, ions have low partition coefficients. Substances 11, 12 and 13 are of special interest in this connection. As Table I shows, the insertion of four carbon atoms into the molecule of dehydracetic acid increases the partition coefficient 2000-fold. However, this is true only of the neutral molecule, which is the form present at pH 3·0. On the other hand, the anion of dehydracetic acid (*i.e.*, the form present at pH 7·3) has an intrinsically lower coefficient, and the insertion of four carbon atoms raises it only 35-fold. Table III shows some further correlations for these substances. At pH 4, a condition under which substances are commonly tested for fungistatic activity, these substances are not appreciably ionised and are highly fungistatic ; at pH 8, these substances are entirely ionised and are only feebly fungistatic. At pH 7·5, where bacteria are commonly tested, they are only feebly antibacterial, presumably for the same reason. These results may shed light on yet other substances believed to be fungistatic but not antibacterial. (Substances 11–13 are such weak metal-binders that we do not wish to claim that metal-binding is responsible for their antifungal action.)

Of all the numerous metal-binding substances evolved by analysis, the majority are ionised at pH 7, and hence unlikely to be antibacterial. Ethylenediamine tetra-acetic acid (versene), which has very high stability constants but is ionised, is an outstanding example of this effect. Substance 10 (Table II) is a further example. Several other ionised reagents, of the most diverse chemical types, have been found to have little or no action on bacteria (Albert *et al.*, 1947). Further development of such substances requires modification of the molecule to repress ionisation.

Thus the rules for improving an unfavourable partition coefficient are known. But it is otherwise with stability constants. In spite of all that has been written on the subject, it is still impossible to assess the magnitude of a stability constant in advance of measurement. Hence many substances must be synthesised and much time spent on titrating them in the presence of metals (solubility permitting) for an occasional substance with high constants to be discovered. Some substances with reputedly high avidities have been found, when measured, to have only modest values. A useful list of stability constants has been made by Irving and Williams (1953), but it must be borne in mind that values obtained in solvents other than water are not relevant to biological problems.

Two other classes of drugs will now be mentioned which bind metals with avidity equalling, or somewhat exceeding that shown by the common amino-

acids. First, isoniazid, and other anti-tubercular hydrazides, which become inactive if modified so that metals can no longer be bound (Cymerman-Craig, Rubbo, Willis and Edgar, 1955), but in which another factor is also involved (Albert, 1956). Again, the tetracyclines (*e.g.*, aureomycin) have considerable affinity for metals (Albert, 1956), particularly for ferric iron (for which the common amino-acids have little affinity). But we find that the tetracyclines kill bacteria so much more slowly than oxine and 2-mercaptopyridine-N-oxide that they cannot act by exactly the same mechanism. *o*-Hydroxyacetophenone (XIV) and 1-hydroxy-2-acetonaphthone (partition coefficient, 32) were examined, because of their structural resemblance to the metal-binding part of the tetracyclines. Both showed a high avidity for ferric ion (*e.g.*, log K′ and K″ of *o*-hydroxyacetophenone were found to be 10·4 and 9·6). However, both lacked antibacterial properties.

Clinical possibilities.

Although little affected by serum, oxine is inactivated by red blood cells (Albert *et al.*, 1947). We have now examined 5-methyl-, 5-chloro-, 7-chloro- and 5 : 7-dichloro-oxines, and 2-mercaptopyridine-N-oxide in the presence of red cells: all were inactivated. The cause of the inactivation of oxine is believed to be a diffusible antagonist liberated by the red cell (Professor G. A. H. Buttle and Dr. W. G. Smith, personal communication).

Professor Buttle has kindly determined the average lethal doses (LD_{50}) in mg./kg. (for mice) of 2-mercaptopyridine-N-oxide (the corresponding values for oxine, obtained at the same time, are given in brackets): oral, 650 (190); subcutaneous, 280 (17); intravenous, 255 (22). Although the mercapto-compound is more active against bacteria than oxine, and has less mammalian toxicity, the inactivation by red cells would limit it to the uses commonly made of oxine, which are principally on the intact mucous membrane.

A more interesting substance for laboratory development may be 2-(2′-hydroxynaphthyl-3′)imidazoline (No. 9, Table II) which is almost unaffected by blood under conditions where the activity of oxine drops to one-twentyfifth.

SUMMARY

The antibacterial action of 2-mercaptopyridine-N-oxide was seen to follow the pattern shown to be typical for oxine, *viz.*, it was rapidly bactericidal, but inactive in the absence of traces of iron, inactive in the presence of iron if traces of cobalt were added, and inactive in concentrated solution.

Stability constants and partition coefficients in conjunction with the above evidence indicate that there is no reason to assume that the 2-substituted pyridine-N-oxides act by sequestering essential heavy metals such as iron. It is suggested that 2-mercaptopyridine-N-oxide enters the cells as a 2 : 1 complex with iron, but that it acts only after this has been broken down.

As with oxine, 2-mercaptopyridine-N-oxide is inactivated by red cells.

A general discussion on the factors which make a chelating substance antibacterial is presented.

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THE RELATIONSHIP BETWEEN THE RESPIRATION AND MULTIPLICATION OF RAT CONNECTIVE TISSUE CELLS *IN VITRO*

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In a previous communication (Harris and Barclay, 1955) a technique was described for measuring the respiration of animal cells *in vitro*, and some observations on the respiration of rabbit macrophages were reported. In the present experiments this technique, with some modification, was used to study the respiration of rat connective tissue cells multiplying *in vitro*.

EXPERIMENTAL

The connective tissue cells were obtained from the hearts of 5-day-old rats. The technique for preparing a homogeneous population of dissociated cells and for measuring their rate of multiplication *in vitro* has already been described (Harris, 1955*a*). The cells were grown in a medium consisting of 50 per cent of bovine serum and 50 per cent of Hanks' solution (Hanks, 1948). Since it had previously been shown (Harris, 1955*b*) that connective tissue cells were unable to survive in serum deficient in cysteine or cystine, L-cysteine hydrochloride was added routinely to the medium at a concentration of 0·001 M. Fig. 1 shows a population of dissociated connective tissue cells multiplying *in vitro*.

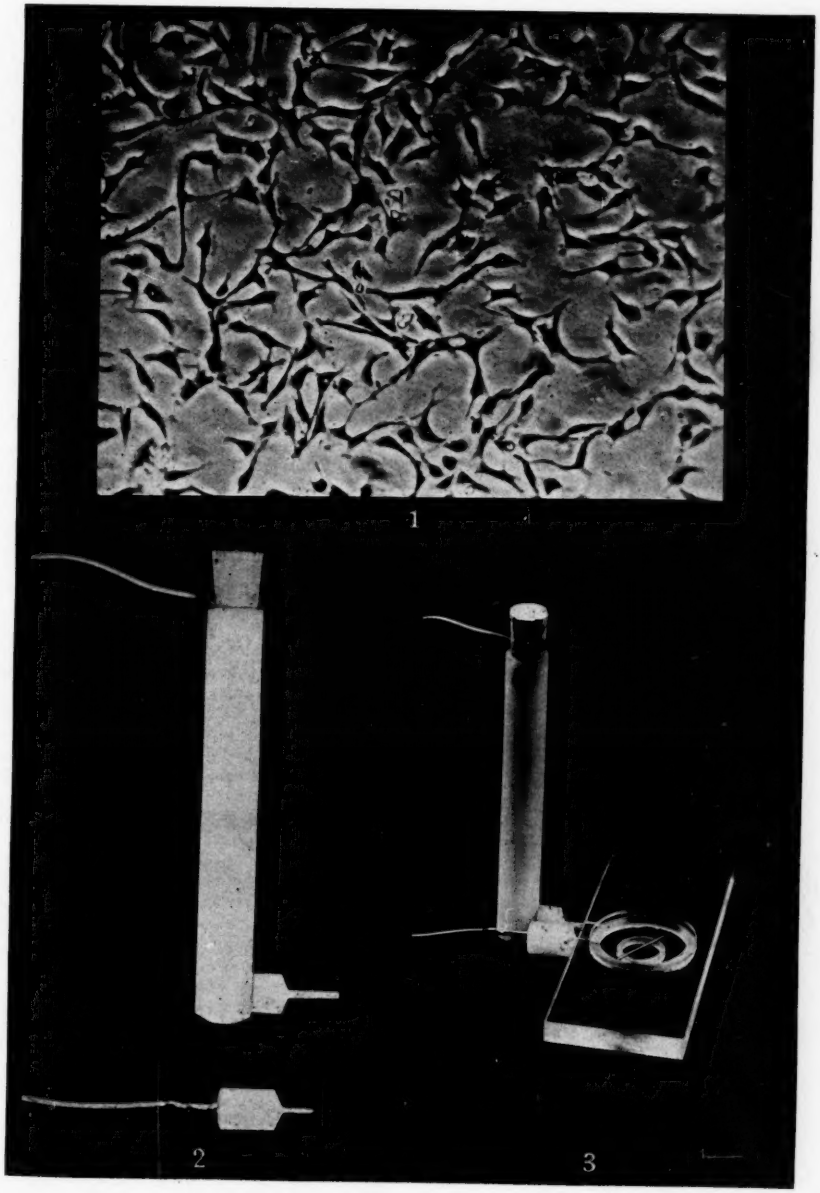
The respiration of the cells was measured in essentially the same way as the respiration of rabbit macrophages (Harris and Barclay, 1955), the oxygen tension of the culture fluid being determined by an adaptation of the platinum-lead circuit devised by Tödt, Teske, Windisch, Heumann and Goslich (1952). The apparatus used in the experiments with macrophages has been improved. The KCl-agar bridges have now been incorporated in tubes of nylon in place of the glass tubes used previously. This has several advantages. The tips of the glass bridges were very fragile and could not easily be drawn out to the same dimensions in each case. The slight variation in the size of the tips resulted in some variation in the resistance of the bridges. This meant that the apparatus had to be re-calibrated with each new bridge. The tips of the nylon bridges are machined to standard dimensions, and thus have a constant resistance when filled with KCl-agar. The nylon bridges are very durable, and the agar in them may be removed by boiling. A single bridge may be used for several months with only one calibration of the apparatus, since renewing the agar does not introduce any significant variation in the resistance of the bridge. A rather more durable nylon cuff was also made for the platinum electrode. Fig. 2 shows the nylon bridge and cuff, and Fig. 3 the culture chamber and electrodes assembled.

The calibration of the electrodes was again carried out by the vacuum flask method previously described (Harris and Barclay, 1955). The relationship between the oxygen tension of the medium and the current produced was of the same general form as that obtained with the glass bridge, although with the rather lower resistance of the nylon bridge the cali

* In receipt of a personal grant from the British Empire Cancer Campaign.

EXPLANATION OF PLATES

FIG. 1.—Dissociated connective tissue cells multiplying *in vitro*.
FIG. 2.—The nylon bridge and the platinum electrode with nylon cuff.
FIG. 3.—The culture chamber and the electrodes assembled.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2
3

bration curve fitted a logarithmic function less closely. Fig. 4 shows the calibration curve obtained with the nylon bridge. The fact that this electrode system gives a curvilinear relationship between O_2 tension and current, whereas the conventional polarograph gives a rectilinear relationship, calls for some comment. There appear to be two main reasons for this curvilinear relationship. The first is that with the narrow agar bridge designed for insertion into the small perspex culture chamber the external resistance in the circuit is not negligible, but influences the form of the O_2 tension/current relationship. The second is that

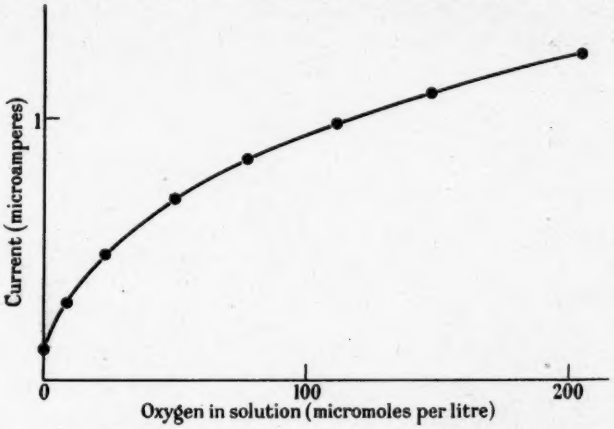

FIG. 4.—Calibration curve showing the relationship between 0_2 tension and current.

the potential developed by the lead half-cell over a certain range is not constant, but falls with diminishing oxygen tension. The significance of these two factors has recently been discussed by Damaschke, Rothbühr and Tödt (1955), who point out that the calibration curve for the apparatus devised by Tödt *et al.* (1952) is curvilinear. In the original report it had been stated that the calibration curve for this apparatus was approximately linear. It seems clear that the form of the relationship between current and O_2 tension, as determined by an electro-chemical circuit, will depend, among other things, on the nature of the metals used and on the precise structure of the electrodes and bridges. The necessity for exact calibration of the electrodes thus becomes evident.

In the experiments previously carried out on macrophages it was not found necessary to make any correction for a blank, as described by Tödt *et al.* (1952). With intermittent readings the current flowed for only a few minutes during the course of an experiment, and since the culture chamber could accommodate up to 750,000 cells, the fall in the oxygen tension of the medium was relatively rapid. Under these conditions any fall in current in the blank could be neglected. This was not always the case with the connective tissue cells. Not more than 200,000 connective tissue cells could be accommodated in the culture chamber, and it was usually necessary to work with smaller populations than this. The fall in the oxygen tension of the medium was thus often a good deal slower than with a dense population of macrophages, and the experiments were consequently of longer duration. The fall in current in the blank had therefore to be taken into account. The blank was obtained for each experiment by repeating the whole procedure in exactly the same way in a chamber containing no cells. Each experimental reading was then corrected against the corresponding reading in the blank.

Chemical methods

Glucose was estimated by the method of Hagedorn and Jensen (1923). Lactic acid was estimated by the enzymic method of Lehmann (1938), the titration being carried out iodimetrically with thiosulphate This method was found to be more accurate than that of Barker and Summerson (1941) which had previonsly been used.

RESULTS

The respiration of the connective tissue cells

Measurements of the respiration of the cells were usually begun 20 hr. after they had been introduced into the chambers. Since the cells gradually increased in size during the period of cultivation, it was necessary to begin experiments at the same stage of growth, if quantitative comparisons of the respiratory rate per cell were to be made. In Fig. 5 the results of an experiment are shown. In this

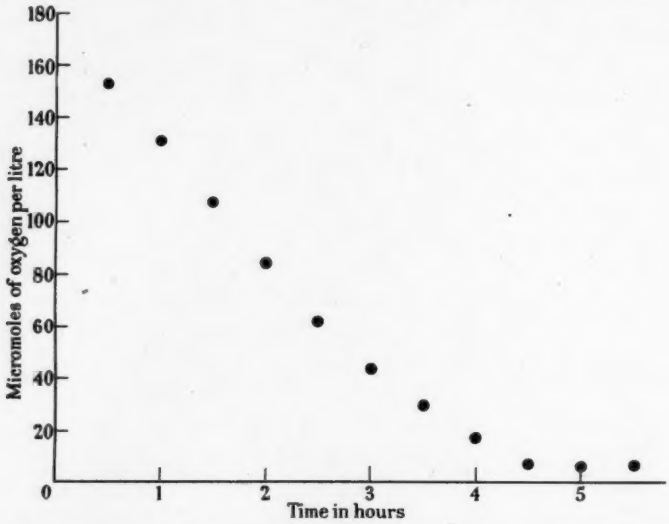

FIG. 5.—The fall in the O_2 tension of the medium in a chamber containing multiplying connective tissue cells.

experiment the chamber contained 200,000 cells, which was about the maximum number possible. It will be seen that the fall in the oxygen tension of the medium was linear over most of the range. At very low oxygen tensions there appears to have been a slight decrease in the rate of fall. The respiratory rate of the cells is thus independent of the oxygen tension of the medium, except perhaps at very low oxygen tensions. The slight fall in respiration at low oxygen tensions was not, however, a consistent finding. In 12 determinations, each begun 20 hr. after the cells had been introduced into the chambers, the mean oxygen uptake was 0·85 micromoles per 10^6 cells per hr. The highest and lowest values were 0·94 and 0·72 respectively.

The multiplication of connective tissue cells under anaerobic conditions

Exhaustion of the oxygen in the medium had no apparent effect on the rate of cell multiplication. A comparison was therefore made between the rate of multiplication of the cells in medium gassed with 5 per cent of CO_2 in oxygen, and in medium gassed with 5 per cent of CO_2 in nitrogen. The latter was not

entirely freed of oxygen by the gassing procedure, but the small amount remaining was rapidly consumed by the cells. Over 4 days, with daily changes of medium, the rate of multiplication of the cells under anaerobic conditions was found to be the same as under aerobic conditions, and the same final population density was reached in both cases. It was thus evident that the connective tissue cell, like the macrophage, is a facultative anaerobe.

The glucose consumption and lactic acid production of connective tissue cells under aerobic and anaerobic conditions

A " Pasteur effect " was demonstrated in the connective tissue cells, as it had been in the macrophages. The consumption of glucose and the production of lactic acid were substantially increased under anaerobic conditions. The results of three typical experiments are given in the Table.

It will be noted that the amount of oxygen taken up by the cells would only account for the combustion of a small proportion of the glucose consumed; even under aerobic conditions most of the glucose was converted to lactic acid.

The effects of some metabolic inhibitors

The multiplication of the cells proceeded at the normal rate in medium freed of oxygen and saturated with carbon monoxide, and also in medium containing 2 : 4 dinitro-phenol in a concentration of 0·0002 M. Since it had been shown that the cells were able to obtain the necessary energy for multiplication in the absence of oxygen, it was not surprising that carbon monoxide and dinitro-phenol were without effect, as these agents interfere only with oxidative metabolic pathways.

Multiplication was inhibited by sodium azide at a concentration of 0·002 M, but the cells remained alive for at least 72 hr. Oxygen consumption was abolished, but glycolysis continued at approximately the normal anaerobic rate. It thus appeared that azide, at this concentration, interfered with the synthetic mechanisms of the cells without impairing glycolysis. This finding is similar to the results obtained by Spiegelman, Kamen and Sussman (1948) using yeast cells. With higher concentrations of azide, glycolysis was also suppressed and the cells gradually died off. Sodium cyanide at a concentration of 0·001 M, and sodium fluoride at the same concentration, inhibited cell multiplication and killed the cells within 48 to 96 hr. Iodoacetamide at a concentration of 0·001 M caused the cells to disintegrate within a few minutes. The results obtained with these metabolic inhibitors are shown in Fig. 6.

TABLE.—*Comparison of the Glucose Consumption and Lactic Acid Production of Connective Tissue Cells under Aerobic and Anaerobic Conditions*

	Oxygen uptake.	Consumption of glucose.	Production of lactic acid.
Aerobic	0·87	1·5	2·2
Anaerobic	—	2·2	3·6
Aerobic	0·85	1·3	2·4
Anaerobic	—	2·0	3·6
Aerobic	0·81	1·5	2·1
Anaerobic	—	2·3	3·2

All values expressed in micromoles per 10^6 cells per hour.

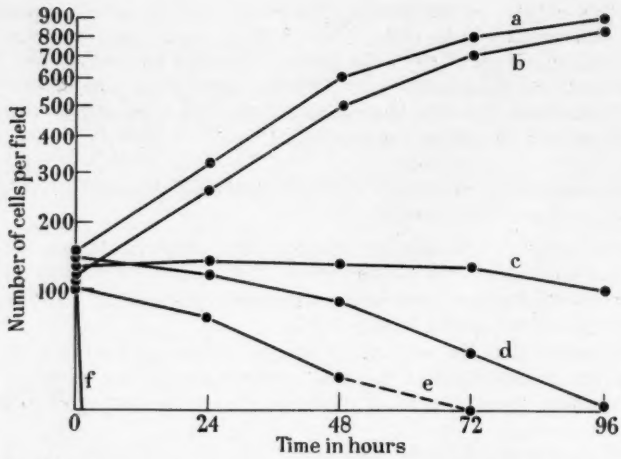

FIG. 6.—The effects of various metabolic inhibitors on the multiplication of the cells.
(*a*) Control: multiplication under aerobic conditions;
(*b*) multiplication in medium free from oxygen and saturated with carbon monoxide;
(*c*) the medium contained sodium azide in a concentration of 0·002 M;
(*d*) „ „ „ sodium fluoride „ „ „ „ 0·001 M;
(*e*) „ „ „ sodium cyanide „ „ „ „ 0·001 M;
(*f*) „ „ „ iodoacetamide „ „ „ „ 0·001 M.

DISCUSSION

These experiments show that rat connective tissue cells are able to multiply at the normal rate under anaerobic conditions. This finding is at variance with some earlier experiments carried out by myself, and with the observations of Goldblatt and Cameron (1953). In the former experiments (Harris, 1955*a*) it was shown that the connective tissue cells could survive for long periods at very low oxygen tensions, but under certain conditions rapid disintegration of the cells occurred. The disintegration in these cases was attributed to acute anoxia. This interpretation was incorrect. The experiments mentioned were carried out before the technique for measuring the oxygen tension of the medium had been developed, and before the critical requirement of the cells for sulphydryl groups had been demonstrated (Harris, 1955*b*). In the present experiments in which cysteine was added to the medium routinely, disintegration of the cells never occurred, and it could be clearly shown that multiplication took place at the normal rate in medium containing no detectable amount of oxygen. Goldblatt and Cameron (1953) reported that if connective tissue cells from the hearts of 5-day old rats were exposed to anaerobic conditions for periods of much longer than half an hour the cells were severely injured and their multiplication was impaired. However, in these experiments, anaerobic conditions were produced by passing a continuous stream of pure nitrogen through the culture tubes. It seems probable that this procedure would cause a very marked rise in the pH of the medium, and it is therefore difficult to be certain that the injury to the cells was due to the anoxia alone. On the other hand Wind (1926), Lipmann (1933) and Laser (1933) all showed

that some multiplication of chick embryo connective tissue cells occurred *in vitro* under strictly anaerobic conditions. Laser pointed out that some of the techniques used to produce anaerobic conditions were injurious to the cells for reasons other than the lack of oxygen. When adequate precautions were taken to avoid these extraneous causes of injury, Laser found that the rate of multiplication of chick embryo connective tissue cells under anaerobic conditions was the same as under aerobic, so far as this could be judged by the size of the emigration zone surrounding the explants. The results of the present experiments with rat connective tissue cells are thus essentially in agreement with the early work of Laser on chick embryo cells.

SUMMARY

By the use of an electro-chemical circuit previously described, measurements were made on the respiration of rat connective tissue cells multiplying *in vitro*. It was found that the cells were able to multiply at the normal rate under completely anaerobic conditions. Certain metabolic inhibitors were tested, and the effects produced by these were consistent with the finding that the multiplication of the cells was not impaired by suppression of oxidative respiration. Under both aerobic and anaerobic conditions most of the glucose consumed by the cells was converted to lactic acid. Under anaerobic conditions the energy necessary for cell multiplication was obtained by an increased rate of glycolysis.

It is a pleasure to thank Miss Marianne Jahnz and Mr. G. J. Wren for careful technical assistance, and Mr. F. Bradley, A.I.B.P., A.R.P.S. for help with the photography.

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THE ALTERATION OF RESISTANCE TO STREPTOCOCCAL INFECTION*

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ENVIRONMENTAL factors, especially the inorganic elements, which influence the virulence of a parasite or the resistance of a host species are still largely undefined both in nature and mechanism of action. The relationship between diphtheria toxin production and the iron concentration of the medium (Pappenheimer and Johnson, 1936), the increased invasiveness of iron-deficient *Brucella suis* (Waring, Elberg, Schneider and Green, 1953), and the observations on the ability of the various metals to lower the natural resistance of mice, guinea-pigs and rats to anthrax infection (Young and Zelle, 1946) prompted an investigation of the effect of the metallic ions in a culture on the virulence of a Group A *Streptococcus pyogenes*. During the course of experiments designed to explore the lethality of this organism when grown in partially inhibitory concentrations of ethylene diamine tetra-acetic acid (EDTA) and various metallic ions, effects due to the action of cobalt and EDTA were observed.

MATERIALS AND METHODS

Tryptose phosphate broth (Difco) was selected for the growth of the organism, since on the basis of turbidity, dry weight, and cellular nitrogen values this medium supported maximum growth.

Blood agar employed for viable counts was prepared from Proteose No. 3 Agar (Difco) by the addition of 5 per cent sterile defibrinated sheep's blood.

All the solutions of metallic ions investigated were prepared from reagent grade salts which conformed to A.C.S. specifications. The EDTA solution was prepared from analytical reagent (Bersworth Chemical Co.).

The strain of streptococcus was obtained through the courtesy of Dr. Rebecca Lancefield and is her culture 6–B410. This organism has a relatively low virulence for mice, and its use therefore allows the detection of small differences in virulence or of host resistance. Many freeze-dried ampoules were prepared from the third broth culture of the organism, and one was used for each of the experiments. In this way the inocula were at the most one broth culture away from a common source. Only mice of the "NAMRU" strain were used throughout this study, and were obtained from the Naval Biological Laboratory where the strain has been maintained and developed for the past fifteen years. This shows highly uniform responses to bacterial infections. Both males and females of $\pm$ 20 g. weight were used.

Viable cell counts were based on the average of seven replicate drop counts of four broth dilutions of the culture. The counts were made after 24 and 48 hr. of incubation at 37°.

Animal experiments

For the ED_{50} titrations five doses of organisms were prepared and groups of 12 mice were injected intraperitoneally with each dose (contained in 0·5 ml.). Any additional materials, such as salts or EDTA, were contained in an additional 0·2 ml. The mice were

* Supported by funds available through a contract between the Office of Naval Research and the Regents of the University.

observed daily and deaths recorded for 14 days. The method of Litchfield and Wilcoxon (1949) was used to obtain: (*a*) the median effective dose, ED_{50}; and (*b*) the factor, $f_{ED_{50}}$, for obtaining 95 per cent confidence limits. In each set of titrations the curves were found to be parallel within experimental error, and the potency ratios, P.R., and their factors, $f_{P.R.}$, could be evaluated:

$$P.R. = ED_{50_1}/ED_{50_2} \text{ where } ED_{50_1} \text{ is the larger.}$$

$$f_{P.R.} = \text{antilog } \sqrt{(\log f_{ED_{50_1}})^2 + (\log f_{ED_{50_2}})^2}$$

Two titrations may be considered significantly different if the value of P.R. exceeds the value of $f_{P.R.}$ The 95 per cent confidence limits of the potency ratio may be calculated as follows:

$$P.R. \times f_{P.R.} = \text{upper limit for } 19/20 \text{ probability}$$

$$P.R./f_{P.R.} = \text{lower limit for } 19/20 \text{ probability.}$$

In studies on the inflammatory response, groups of five mice were injected intraperitoneally with approximately one ED_{50} dose of streptococci and either 0·2 mg. ($6{\cdot}87 \times 10^{-7}$ M) of cobalt nitrate in solution or an equal volume of saline. At intervals after the injections the mice were killed with ether and the inflammatory exudate studied.

Rabbit anti-streptococcal serum was prepared by repeated intravenous injections of the homologous streptococci killed by exposing the saline suspension to 56° for 1 hr. The serum thus obtained agglutinated 38×10^6 streptococci at a dilution of 1/1280 in a total volume of 1 ml. It yielded 0·136 mg. precipitable nitrogen per ml. when reacted with purified C polysaccharide.

Suspensions of sensitised streptococci were prepared by mixing 0·1 ml. of packed cells in 1·5 ml. saline with an equal volume of antiserum, mixing and incubating at 37° for 1 hr. The cells were sedimented again by centrifugation and the antibody absorbed from another aliquot of serum in the same way. The cells were again spun down, re-suspended in saline and used immediately.

Mouse complement was obtained by pooling the blood obtained by the decapitation of 50 mice. This blood was kept in an ice bath during collection. After clotting, the cells were spun down in a refrigerated centrifuge, and the serum removed. Complement so prepared was used on the same day.

Exudates were collected from the peritoneal cavities after injecting 1 ml. of saline and sacrificing the animals by ether anaesthesia.

The number of leucocytes per cmm. was determined in an AO Spencer Bright Line Haemacytometer. No dilution of the fluid was made. In all cases only counts in which the numbers of leucocytes per each of the four 0·04 mm.^{2} areas agreed $\pm$ 10 were recorded. New preparations were made by refilling the chamber until this degree of replication was attained.

The different kinds of cells present in the inflammatory exudate were counted from smears prepared with Wright's Stain. The per cents expressed in the experimental section refer to the number of cells of a given type per 100 cells observed. The number of cells showing phagocytised streptococci were recorded at the same time.

Because of the variety of transitional mononuclear cells that may be present in the mouse peritoneum, no attempt was made to classify this cell group further. Under the name of mononuclear leucocytes were included both small lymphocytes and large mononuclear cells of the monocyte type. Since it was technically possible to obtain only small samples the Student *t* test was used to determine significance of differences (Fisher, 1946).

RESULTS

Virulence of Organisms Grown in a Medium in which Metallic Ion Concentration was Controlled with EDTA

The lethality in 14 days for mice of cells grown in broth containing $1{\cdot}45 \times 10^{-3}$ M EDTA and that concentration of each metal which antagonised the inhibition of

EDTA was determined. Only small differences in the ED_{50} were found. However, it was observed that the presence of either cobalt or manganese appeared to exert a marked influence on survival time. The response to dosage data in Table I shows that all fatally infected mice injected with organisms grown in cobalt + EDTA were dead by the fourth day and that more than 50 per cent of these mice were dead by the second day. The titration involving manganous chloride showed a similar picture although the time to death was not shortened as markedly. The titrations of streptococci grown in the presence of other ion-EDTA combinations showed more variation in survival time; deaths occurred over a 14-day observation period. The titration of organisms grown in plain broth gave the usual type of result.

TABLE I.—*Lethality Exhibited over 14-Day Period of Infection*

Organisms grown from broth + $1 \cdot 37 \times 10^{-3}$ M cobalt nitrate and $1 \cdot 45 \times 10^{-3}$ M EDTA.

Dose ($\times 10^6$).	Days. 1	2	3	4	5	6	7	8	9	10	11	12	13	14
6·8	—	9/12*	—	—	—	—	—	—	—	—	—	—	—	9/12
3·4	—	2/12	4/12	5/12	—	—	—	—	—	—	—	—	—	5/12
1·7	—	4/12	—	—	—	—	—	—	—	—	—	—	—	4/12
0·85	—	—	—	—	—	—	—	—	—	—	—	—	—	0/12
0·68	—	—	—	—	—	—	—	—	—	—	—	—	—	0/12
Organisms grown in broth + $1 \cdot 35 \times 10^{-3}$ M manganous chloride and $1 \cdot 45 \times 10^{-3}$ M EDTA.														
6·6	2/12	8/12	9/12	10/12	—	—	—	—	—	—	—	—	—	10/12
3·3	—	7/12	9/12	—	10/12	—	—	—	—	—	—	—	—	10/12
1·65	—	3/12	5/12	6/12	7/12	8/12	—	—	—	—	—	—	—	8/12
0·825	—	1/12	—	2/12	—	3/12	4/12	—	—	—	—	—	—	4/12
0·66	—	—	—	1/12	—	2/12	—	—	—	—	—	—	—	2/12
Organisms grown in plain broth.														
4·35	1/12	2/12	3/12	4/12	7/12	—	—	8/12	—	—	—	—	—	8/12
2·175	—	1/12	—	2/12	—	—	3/12	—	—	4/12	5/12	—	—	5/12
1·088	—	—	—	1/12	—	—	—	—	—	2/12	—	—	—	2/12
0·544	—	—	—	—	—	—	—	—	—	—	—	—	—	0/12
0·435	—	—	—	—	—	—	—	—	—	—	—	—	—	0/12

* Ratios in body of Table are cumulative dead/number animals at risk for each day of observation.

Further study was devoted to the question of whether a real difference in the survival time existed and to the question of whether growth of the streptococcus in an environment generally deficient due to the action of EDTA or in an environment rich in cobalt was responsible for the reduced survival time or whether alteration in survival time was due to an action of EDTA or cobalt nitrate or both on the host.

Effect of Cobalt and EDTA on Infection by Streptococci Grown in Normal Medium

The organism was grown in broth and either EDTA or cobalt nitrate, or both were injected intraperitoneally immediately after the injection of organisms. The doses of these substances corresponded to the amount that would be contained in the supernatant from a culture grown in the presence of $1 \cdot 37 \times 10^{-3}$ M cobalt nitrate and $1 \cdot 45 \times 10^{-3}$ M EDTA, *i.e.*, $6 \cdot 87 \times 10^{-7}$ moles of cobalt nitrate and $7 \cdot 25 \times 10^{-7}$ moles of EDTA. Organisms from a medium containing these amounts of EDTA and cobalt nitrate were injected into other mice for comparison. Diluent

broth contained both EDTA and cobalt nitrate in the same concentrations. Organisms grown in the partially inhibitory concentration of $3{\cdot}22 \times 10^{-5}$ M EDTA were injected into another group of mice using as diluent broth containing the same concentration of EDTA. Organisms grown in broth containing $8{\cdot}56 \times 10^{-4}$ M cobalt nitrate, a partially inhibitory level of this substance, were also studied. Dilutions were made in the same concentration of cobalt nitrate. In order better to compare the abilities of cobalt and EDTA to shorten the survival time the ED_{50} values were calculated from the number of deaths occurring in only the first 4 days although observation was continued to the 14th day. The ED_{50} values are summarised in Table II.

TABLE II.—*Response of Mice to Injection of Streptococci, EDTA and Cobalt Nitrate*

Organisms grown in :	Calculation at 4 days. ED_{50}.	$f_{ED_{50}}$.	Calculation at 14 days. ED_{50}.	$f_{ED_{50}}$.
Broth	$9{\cdot}1 \times 10^6$ (4·7–17·8)	1·95	$3{\cdot}0 \times 10^6$ (1·8–4·9)	1·65
Broth, followed by $Co(NO_3)_2$ injection ($6{\cdot}87 \times 10^{-7}$M)	$1{\cdot}5 \times 10^6$ (0·88–2·6)	1·70	$0{\cdot}91 \times 10^6$ (0·54–1·55)	1·70
Broth, followed by EDTA injection ($7{\cdot}25 \times 10^{-7}$M	$1{\cdot}5 \times 10^6$ (0·88–2·6)	1·70	$0{\cdot}72 \times 10^6$ (0·41–1·26)	1·75
Broth, followed by $Co(NO_3)_2$ and EDTA injection (concentrations as above)	$0{\cdot}42 \times 10^6$ (0·20–0·88)	2·10	$0{\cdot}25 \times 10^6$ (0·10–0·60)	2·40
Broth + $Co(NO_3)_2$ ($8{\cdot}56 \times 10^{-4}$M)	$3{\cdot}6 \times 10^6$ (1·6–8·3)	2·25	$1{\cdot}4 \times 10^6$ (0·78–2·5)	1·80
Broth + EDTA ($3{\cdot}22 \times 10^{-5}$M)	$3{\cdot}6 \times 10^6$ (1·6–8·3)	2·30	$2{\cdot}3 \times 10^6$ (1·2–4·4)	1·90
Broth + $Co(NO_3)_2$ + EDTA (concentrations as above)	$3{\cdot}6 \times 10^6$ (1·3–10·1)	2·80	$0{\cdot}49 \times 10^6$ (0·20–1·2)	2·40

A reduced survival time was observed where either cobalt nitrate, EDTA or both of these substances were injected immediately following an injection of broth-grown organisms. These observations indicated that the effects of cobalt nitrate and EDTA were directed primarily against the host. Table III reveals that four test situations were characterised by ED_{50} values significantly different from that of broth-grown organisms. Growth in the presence of $8{\cdot}56 \times 10^{-4}$ M cobalt nitrate or in the presence of $3{\cdot}22 \times 10^{-5}$ M EDTA did not produce organisms with increased killing power even when these substances in the above concentrations were injected with the dose of cells. All of the other test situations yielded ED_{50} values significantly lower than those obtained with broth-grown cells. Both EDTA and cobalt nitrate were capable independently of lowering the ED_{50} on injection. There was no significant difference in the relative potency of EDTA and cobalt nitrate. The possibility that their effects were additive was indicated by the significant lowering of the ED_{50} when both were injected. Prior growth of the organism in the presence of the effective levels of EDTA and cobalt was not necessary considering the lack of significant difference between ED_{50} values for such cells and those grown in broth followed by the injection of EDTA and cobalt. Therefore, the effect of cobalt and EDTA is on the host. The doses of EDTA and cobalt nitrate employed produced no deaths either alone or combined when tested in groups of twelve mice.

Study of chain length, staining properties, and colonial morphology of the streptococci gave no indication that these properties were in any way affected by growth in the presence of EDTA and/or cobalt nitrate.

TABLE III.—*Estimate of Relative Virulence Determined at Four Days*

ED_{50_2} / ED_{50_1}	Streptococci grown in broth.	Broth followed by $Co(NO_3)_2$ injection.	Broth followed by EDTA injection.	Broth followed by $Co(NO_3)_2$ and EDTA injections.	Broth + $Co(NO_3)_2$.	Broth + EDTA.
Broth followed by $Co(NO_3)_2$ injection	P.R. 6·07*[1]	—	—	—	—	—
	$f_{P.R.}$ 2·34	—	—	—	—	—
Broth followed by EDTA injection	P.R. 6·07*[2]	1·00	—	—	—	—
	$f_{P.R.}$ 2·34	2·11	—	—	—	—
Broth followed by $Co(NO_3)_2$ and EDTA injections	P.R. 21·70*[3]	3·57*	3·57*	—	—	—
	$f_{P.R.}$ 2·48	2·48	2·48	—	—	—
Broth + $Co(NO_3)_2$	P.R. 2·53	2·40	2·40	8·57*	—	—
	$f_{P.R.}$ 2·85	2·64	2·64	3·00	—	—
Broth + EDTA	P.R. 2·53	2·40	2·40	8·57*	1·00	—
	$f_{P.R.}$ 2·85	2·64	2·67	3·50	3·20	—
Broth + EDTA and $Co(NO_3)_2$	P.R. 2·53*[4]	2·40*	2·40*	8·57*	1·00	1·00
	$f_{P.R.}$ 2·43	2·20	2·20	2·57	2·72	2·76

* Significantly different.

[1] 95 per cent confidence limits 2·59–14·20
[2] 95 " " " 2·59–14·20
[3] 95 " " " 8·75–50·78
[4] 95 " " " 1·04– 6·15

Effect of Metallic Ions on Natural Defence Mechanism of the Host

When it appeared that reduced survival time was the result of an action by cobalt on the host, a series of determinations were made to define the limitations of the dose of metallic ion. A comparison of the relative potencies showed that there was no significant difference between the titrations involving no cobalt, 0·020 mg., and 0·002 mg. cobalt nitrate. The administration of 0·200 mg. cobalt nitrate ($6{\cdot}87 \times 10^{-7}$ moles) again produced the observed effect.

The level of 0·2 mg. was taken as a standard dose for determining the specificity of cobalt in the enhancement phenomenon. Equimolar doses of other metallic ions, $FeSO_4$, $CuSO_4$, $ZnSO_4$, $MnCl_2$, $MgSO_4$, and $CaCl_2$, were injected intraperitoneally immediately following the organisms. One set of animals received organisms followed by the injection of 0·2 ml. of saline for comparison. Analysis of the data revealed that only cobalt nitrate was capable of reducing the survival time as expressed as a lower ED_{50} at 4 days. It may be assumed, therefore, that the phenomenon is specific for cobalt nitrate.

When cobalt nitrate was compared with an equimolar solution of cobalt sulphate, no significant difference in the potency was detected. Both salts significantly lowered the ED_{50} when compared with the results on mice which received saline in place of a cobalt salt.

Total leucocyte counts.—The response of mice to the injection of one ED_{50} dose of streptococci followed by the injection of 0·2 mg. cobalt nitrate ($6{\cdot}87 \times 10^{-7}$

moles) or an equal volume of saline was determined. Examination of the means of the counts against time showed only small differences in the total number of leucocytes. Therefore, cobalt does not directly affect the inflammatory response by inhibiting the infiltration of leucocytes.

Differential leucocyte counts.—The changes in the percentages of the mononuclear and polymorphonuclear cells at various times during the early hours of the infection were determined. There were only small differences in the types of cells present at the primary site of infection.

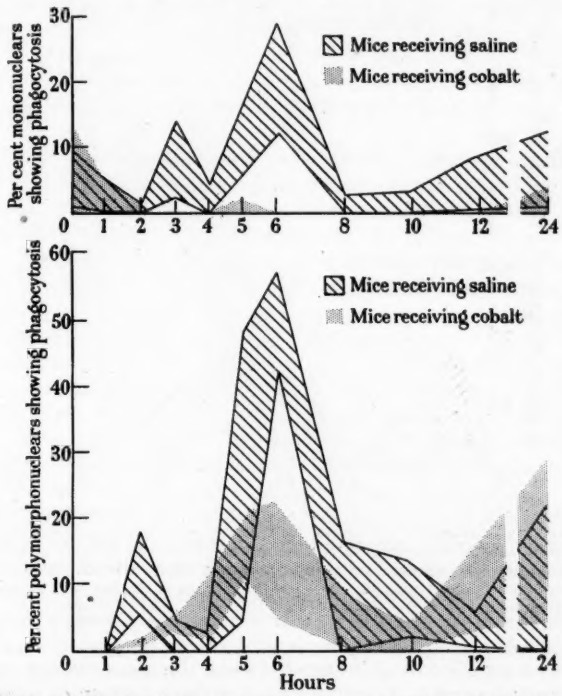

FIG. 1.—Percentages of mononuclear and polymorphonuclear leucocytes showing phagocytosis. Five mice were examined after each time interval. The means of the counts were calculated and the areas between plus and minus two standard errors were plotted. The significance of difference between divergent areas was determined by the use of the Student *t* test for the significance of difference between the means of small samples (Fisher, 1946).

The percentages of the mononuclears and polymorphonuclears which phagocytised streptococci are depicted in Fig. 1, and plots of the means plus and minus two standard errors of the counts against time are included. There was no significant difference between the mononuclears of the cobalt-treated and untreated mice until the 3rd hour of infection, at which time the mononuclears in the cobalt-treated animals ceased ingesting the parasite. This condition persisted, for the most part, until the 10th hour of infection. Thus it appeared that the ability of the mononuclear cells to phagocytise streptococci was inhibited at this stage of the

infection when cobalt had been injected. The polymorphonuclears also appeared to be inhibited between the 5th and 8th hours after infection but were not inhibited as completely as were the mononuclear cells.

Viable counts of streptococci.—The results of these counts are summarised in Fig. 2 which includes a plot of the means of the counts $\pm$ 2 S.E. The times at

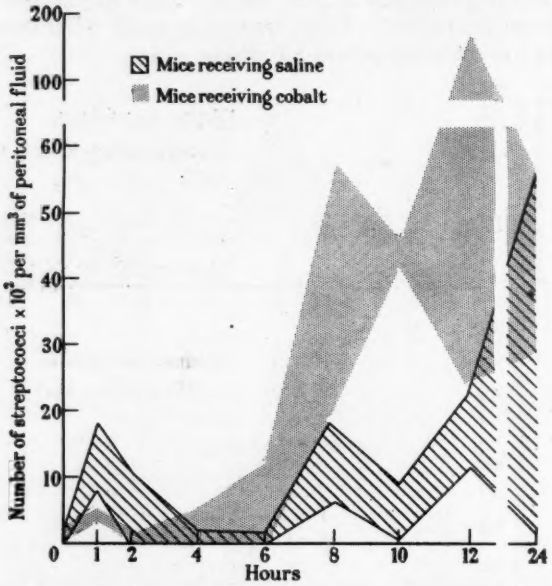

FIG. 2.—Streptococcal content of peritoneal fluid at different stages of infection. Five mice were examined after each time interval. The means of the counts were calculated, and the areas between plus and minus two standard errors were plotted. The significance of difference between divergent areas was plotted by the use of the Student *t* test for the significance of difference between the means of small samples (Fisher, 1946).

which there was a significant difference between the means of the counts obtained from the two groups were 1, 8 and 10 hr. after the injection of organisms. At the end of the first hour the number of viable streptococci present in the peritoneal cavities of untreated animals was significantly higher than the number present in cobalt-treated animals. This may be due to a toxic action of the cobalt. However, by the 6th hour the action of cobalt on the phagocytosis picture was most pronounced and after this time the streptococci increased steadily in number in the cobalt-treated animals. The lack of significant difference between the means of the counts after the 10th hour appears to be due mainly to the great variation in conditions existing in the peritoneal cavities of the various mice. Certain cobalt-treated mice gave very high counts, others fairly low. Since the dose of streptococci injected was only one ED_{50} dose, such variation was to be expected when the effect of the cobalt was diminishing. The time at which this effect started to diminish appeared to be around the 10th hour if one considered the degree of phagocytosis.

Cultures of heart blood from mice killed at the various times all showed the presence of streptococci. Even after the first hour of infection there were numerous streptococci present in the blood. Thus there was an even greater population of organisms showered into the blood in those mice receiving cobalt.

Effect of Specific Antibody on Cobalt Enhancement

When it appeared that cobalt produced its effect of shortening survival time by inhibiting the phagocytic abilities of mononuclear and polymorphonuclear leucocytes, experiments were performed to determine whether this effect could be overcome by providing substances which have been found to aid phagocytosis. In this way it might be possible to determine whether cobalt was interfering with some preliminary sensitisation of the streptococcus or was affecting the phagocytes themselves.

The first of such substances tested was antibody prepared in rabbits by immunisation with heat-killed streptococci. The antiserum was injected intraperitoneally and determinations of the phagocytic ability of the leucocytes were made an hour later. There was no significant increase in the percent of leucocytes showing phagocytosis. A second experiment using antibody was performed in which a heavy suspension of streptococci previously sensitised with antibody was injected intraperitoneally and an hour later counts of the cells showing phagocytosis were made. The injection of sensitised cells significantly raised the percentage of leucocytes showing engulfed organisms only in polymorphonuclears from non-cobalt treated mice. The percentage of mononuclear leucocytes showing phagocytosis was not affected nor were the phagocytic abilities of the leucocytes from cobalt-treated mice. The cobalt produced its effect by inhibiting phagocytosis as such. Whether this was due to a change induced in the phagocytic cell or to a change in its external environment was not further determined.

Similar determinations of the phagocytic capacity of leucocytes were made following the injection of complement in the form of fresh mouse serum. This substance had no effect on cobalt inhibition and also no effect on the phagocytic capacity of leucocytes from non-cobalt treated mice.

The possibility that Co^{++} induced *in vivo* a change in the streptococci, rendering them less susceptible to phagocytosis, was suggested by the findings of Burrows and Bacon (1954) on *Pasteurella pestis*.

Heavy suspensions of washed streptococci were obtained from the peritoneal cavities of cobalt-treated mice 8–10 hr. after injection. These organisms upon reinjection into normal mice were phagocytised to the same degree as were heavy suspensions of washed streptococci obtained from the peritoneal cavities of non-treated mice and similarly injected into other mice. It does not appear therefore that cobalt led to the appearance of populations of organisms inherently resistant to phagocytosis.

To further test the hypothesis that cobalt produced its effect by inhibiting the phagocytic capacity of leucocytes a twice-washed suspension of diluted India ink particles was injected in place of streptococci. Cobalt nitrate solution or an equal volume of saline was injected immediately following and differential counts performed on the peritoneal fluid 6 hr. later. The number of mononuclear cells showing phagocytised carbon particles was significantly lower in the cobalt-treated mice. Polymorphonuclear leucocytes from the five cobalt-treated mice

showed a lower degree of phagocytosis than did this type of cell from non-treated mice but the probability that there was a significant difference was just below the 95 per cent level.

Cobalt also did not affect the bactericidal action of whole fresh mouse blood.

DISCUSSION

The enhancement of streptococcal infection by cobalt is evidenced by a small yet significant decrease in the ED_{50} when it is determined after the fourth day of infection. While there is also a significant reduction in the ED_{50} at the fourteenth day (see Table IV), cobalt most characteristically causes a shortening of the time to death. The action of cobalt is directed towards the phagocytic cells. Of such cells the mononuclear leucocytes are most affected. Due to this inhibitory action the streptococci are allowed to multiply freely in the peritoneal cavity and are showered continually into the general circulation. The injection of cobalt is then outwardly equivalent to the injection of a larger number of streptococci.

TABLE IV.—*Estimate of Relative Potency Determined at Fourteen Days*

ED_{50_2} / ED_{50_1}	Streptococci grown in broth.		Broth followed by $Co(NO_3)_2$ injection	Broth followed by EDTA injection.	Broth followed by $Co(NO_3)_2$ and EDTA injections.	Broth + $Co(NO_3)_2$.	Broth + EDTA.
Broth followed by $Co(NO_3)_2$ injection	P.R.	3·29*[1]	—	—	—	—	—
	$f_{P.R.}$	2·06	—	—	—	—	—
Broth followed by EDTA injection	P.R.	4·17*[2]	1·27	—	—	—	—
	$f_{P.R.}$	2·12	2·16	—	—	—	—
Broth followed by $Co(NO_3)_2$ and EDTA injections	P.R.	12·0*[3]	3·64*	2·88*	—	—	—
	$f_{P.R.}$	2·73	2·77	2·82	—	—	—
Broth + $Co(NO_3)_2$	P.R.	2·14	1·54	1·95	5·60*	—	—
	$f_{P.R.}$	2·16	2·20	2·25	2·86	—	—
Broth + EDTA	P.R.	1·31	2·53*	3·19*	9·19*	1·64	—
	$f_{P.R.}$	2·25	2·28	2·34	2·95	2·37	—
Broth + EDTA and $Co(NO_3)_2$	P.R.	6·12*[4]	1·86	1·47	1·96	2·86	4·69*
	$f_{P.R.}$	2·72	2·77	2·82	3·45	2·86	2·96

* Significantly different.

[1] 95 per cent confidence limits 1·59– 6·78
[2] 95 „ „ „ 1·97– 8·84
[3] 95 „ „ „ 4·39–32·76
[4] 95 „ „ „ 2·25–16·65

The results of these experiments suggest a relation to a series of reported observations. Dubos (1953) has shown that keto-acids enhance the viability of *Mycobacterium tuberculosis* and staphylococci in culture and has suggested that these substances may antagonise the natural bactericidal action of the lactic acid normally a part of the inflammatory exudate. Berry and his colleagues (Berry and Mitchell, 1953*a*, *b*; Berry, Merritt and Mitchell, 1954; Berry, Ehlers and Mitchell, 1954) reported that the injection of various Krebs cycle inhibitors and intermediates may enhance bacterial infection and shorten the survival time of mice. That cobalt and EDTA, two substances which have been shown

here to enhance infection, may directly interfere with tricarboxylic acid metabolism is indicated by the experiments of Levy, Levison and Schade (1950) and Tyler (1955). Cobalt is inhibitory for rat liver and kidney succinic dehydrogenase (Levy *et al.*, 1950). EDTA has been shown to cause an accumulation of oxalo-acetic acid during succinate oxidation by whole tissue and mitochondrial suspensions. Cobalt has been shown to be an effective inhibitor of other enzymes, cytochrome oxidase (Levy *et al.*, 1950) and red blood cell reduction systems (Shu, Ley and Grant, 1954) which might indirectly lead to keto-acid accumulations by general interference with oxidation or the creation of anoxia.

These observations suggest that one mechanism worthy of further investigation for the cobalt effects,which were shown to be additive to those of EDTA, is that an interference with keto-acid metabolism of the phagocytic cells or other tissue elements occurs on injection of cobalt, allowing acids of this type to accumulate. Such a metabolic disorder might lead to inactivation of the ingestion mechanism of the phagocytic cells. When this occurs in the immediate vicinity of streptococci, growth of the latter is enhanced in consequence of the failure of the phagocyte to act, and accumulation of the keto-acids may further interfere with the natural bactericidal action of lactic acid normally a part of the inflammatory exudate. This sequence of events would explain the present findings.

SUMMARY

In a study of the effect of metallic ions on a Group A streptococcus the most pronounced aspect of an ion effect was the shortening of the time to death of those mice which were fatally infected. In this respect cobalt nitrate had the greatest influence among the salts studied. The effect of cobalt appeared to be directed towards a lowering of host resistance. EDTA acted in a similar manner. Both cobalt sulphate and nitrate produced the effect, and the reduction in survival time was not a general effect but was restricted to cobaltous ion.

Examination of the inflammatory response to streptococcal infection when cobalt is present or absent revealed that cobalt appears to produce its effect by inhibiting phagocytosis. The mononuclear leucocytes are particularly affected. Speculations are made as to the mechanism whereby cobalt affects phagocytic cells.

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WHOLE BODY IRRADIATION AND THE IDEA OF STRESS

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SINCE Selye first suggested that a sequence of changes in the secretions of endocrine glands was the final common path by which a large variety of stimuli affected the organism, the idea of stress has been used enthusiastically as an explanation, perhaps without sufficient attention being given to its precise definition (Lancet, 1951). Whole body irradiation is always included in lists of stressing stimuli and a considerable amount of evidence has been accumulated which suggests that a near-lethal dose of whole body irradiation does indeed stimulate the adrenal gland of the rat: there is a temporary reduction in adrenal cholesterol a few hours after irradiation (Patt, Swift, Tyree and John, 1947; Nims and Sutton, 1954), a rise in liver glycogen in fasted animals (North and Nims, 1949; McKee, 1952; Nims and Sutton, 1954), and later a considerable increase in the weight of the adrenal glands (Patt *et al.*, 1947; Anderson, Blaschko, Burn and Mole, 1951). These changes are prevented by hypophysectomy (Patt, Swift, Tyree and Straube, 1948; Nims and Sutton, 1954). Yet irradiation, unlike most injurious agents, does not produce overt injury or even stimulate sense-organs; indeed it is simple to arrange dummy and real irradiations so that an individual animal or person is unaware of being irradiated or injured. In these circumstances how can the irradiated organism be under stress except in some unconscious or para-Freudian sense of that word? Whole body irradiation may thus be a test case of the idea that specific effects follow general non-specific injury, and the evidence that it stimulates the adrenal glands has been re-examined and found wanting.

The most direct method for investigating adrenal activity, the estimation of compound F in the plasma, is not sensitive enough to be usable on the size of blood sample obtainable from a single rat (Bayliss, R. I. S., 1954, personal communication). There are gross disturbances in water metabolism of the rat after whole body irradiation with lethal doses, polydipsia and polyuria in the first twenty-four hours (Edelmann, 1949), and dehydration and oliguria on the third day, so that changes in urinary steroids are not likely to be easily interpreted. Eosinophile counts inevitably fall to very low levels because of the gross radiation damage to the bone marrow. Thus the only method applicable seemed to be the comparison of the level of liver glycogen in fasted irradiated rats with that in unirradiated controls, the metabolic change used in the bio-assay of the glucogenic cortical steroids (Dorfmann, 1954).

METHODS

A full account of the analytical and operative techniques is given elsewhere (Herbert and Mole, 1956). Each comparison was made between litter-mates treated similarly except that one was given a dummy irradiation, the other a real irradiation. Irradiation was with X-rays

at 240 kv, 43 R/min., HVL 1·2 mm. Cu and in a field uniform to ± 3 per cent. Bilateral adrenalectomy and sham operations were done under amytal anaesthesia 24 hr. before killing the animals for the determination of blood sugar and liver glycogen. Evidence for the completeness of the adrenalectomies is given elsewhere (Herbert and Mole, 1956). Cortisone acetate, when administered, was given in five simultaneous widely-spaced subcutaneous injections immediately after the end of the operation for removal of the adrenals. Bilateral orchidectomy was carried out 2–3 weeks before irradiation. Rats were starved for 48 hr. before killing: they had been kept for at least 3 weeks at an air temperature of 27 ± 1° at which temperature their food intake is minimal and the effects of starvation minimised.

RESULTS

A dose of 200 R of X-rays is between one-quarter and one-third of the LD_{50} for the rats used in these experiments, and its administration is followed by little visible change in the appearance or behaviour of the animals. However, 24 hr. after 200 R of whole body X-irradiation there was a six- to tenfold rise in liver glycogen in intact and sham-operated rats followed by a return towards normal a day later (Table I). This rise in liver glycogen was prevented by removal of the adrenal glands shortly after irradiation, in agreement with the earlier reports already cited. However, if cortisone acetate was injected immediately after adrenalectomy, the rise in liver glycogen following irradiation was indistinguishable from that occurring in intact or sham-operated rats. In the adrenalectomised rats there can be no question of any increased adrenal secretion: the irradiated and control animals were given the same amount of hormone. The dose of hormone used was "physiological"; it was just sufficient to raise the blood sugar of adrenalectomised control rats from its normal depressed value to a level equal to that of intact or sham-operated controls (Table II). The rise in liver

TABLE I.—*Rise in Liver Glycogen Concentration after* 200R *Whole Body X-irradiation*

	24 hr. after irradiation.		48 hr. after irradiation.	
	No. of rats.	Ratio: $\frac{\text{irradiated}}{\text{control.}}$	No. of rats.	Ratio: $\frac{\text{irradiated}}{\text{control.}}$
		Mean ± S.E.		Mean ± S.E.
Intact	9	10·2 ± 3·7	9	1·8 ± 0·4
Sham-operated	6	6·3 ± 1·8	7	3·2 ± 1·2
Adrenalectomised (no cortisone)	3	1·1 ± 0·2	..	..
Adrenalectomised (1·25 mg. cortisone)	5	7·8 ± 4·2*	3	0·9 ± 0·3
Castrated	3	6·3 ± 1·4	..	..

The liver glycogen concentration in each irradiated rat was expressed as a multiple of its own litter-mate control: these were kept in the same cage, treated in the same way except for the actual irradiation, and killed within a few minutes of each other.

* Variance analysis showed that the glycogen level in the irradiated rats was significantly greater than in the litter-mate controls ($P < 0·02$).

TABLE II.—*Blood Sugar (mg./100 ml. mean ± S.E.) after* 200R *Whole Body X-irradiation*

	Hours after irradiation.		
	0.	24.	48.
Intact	92 ± 2	117 ± 6	94 ± 5
Sham-operated	91 ± 3	108 ± 4	100 ± 5
Adrenalectomised (no cortisone)	67 ± 3	87 ± 5	..
Adrenalectomised 1·25 mg.. cortisone)	86 ± 2	109 ± 12	90 ± 5
Castrated	93 ± 2	110 ± 6	..

glycogen in irradiated rats is therefore not evidence of increased adrenal secretion, as has been supposed, but in a sense is due to the irradiated animals using cortisone more efficiently. Additional results show that this was also true in females and after 400, 600 and 1000 R.

The reason why the irradiated animal apparently uses cortisone more efficiently is suggested by the changes in blood sugar shown in Table II. Irradiation always caused a rise in blood sugar whether the rats were intact or adrenalectomised or adrenalectomised and given cortisone. The relation between blood sugar and liver glycogen is such that unless the blood sugar exceeds a certain threshold value no liver glycogen is laid down (Herbert and Mole, 1956). When the blood sugar exceeds this threshold, glycogen is laid down in rapidly increasing amounts as the blood sugar rises. Thus in the adrenalectomised rat although the blood sugar rose after irradiation (Table II) it did not exceed this threshold. In the adrenalectomised rat as in intact or sham-operated rats, however, the increase produced by irradiation brought the blood sugar above the point where glycogen deposition in the liver begins.

As shown in Tables I and II orchidectomy did not affect the response to irradiation.

DISCUSSION

A standard line of argument in considering stress phenomena has been to take increased adrenal activity as proved when a response is prevented by adrenalectomy (or hypophysectomy) and when it can be imitated in the intact animal by injection of adrenal hormone. The results presented show that this is not necessarily a legitimate argument when, as may often be the case, the response depends on the interaction of adrenal hormone and some other bodily constituent the concentration of which is also dependent in part on the available hormone. The rise in liver glycogen after irradiation was prevented by adrenalectomy, and a rise in liver glycogen in unirradiated animals is produced by injection of adrenal hormone. Nevertheless the increased liver glycogen after irradiation was due to a change in the other bodily constituent, the blood sugar, not to an increase in adrenal cortical activity. The possibility of this kind of interaction should be critically considered whenever increased adrenal activity is in question.

It has recently been shown that many metabolic changes occur in adrenalectomised patients which in the past were considered to be due to increased adrenal activity (Robson, Horn, Dudley and Stewart, 1955; Mason, 1955). Earlier, Ingle, Ward and Kuizenga (1947) had investigated post-traumatic nitrogen excretion in adrenalectomised rats also maintained on a constant dose of adrenal hormone. They concluded that the adrenal glands played only a permissive rôle: an increased secretion of adrenocortical steroids was not the cause of the metabolic change but the presence of some hormone was necessary for it to occur. The same interpretation might well have applied to the changes in liver glycogen after irradiation, but the blood sugar measurements showed that it was not even necessary to postulate a limited permissive rôle for the adrenal glands to explain metabolic changes found after this form of injury.

A major part of the evidence that whole body irradiation causes increased adrenal activity has been shown to have been misinterpreted, and other evidence is also susceptible of other interpretations, as will be discussed elsewhere. Mean-

while it is suggested that the rise in blood sugar after irradiation is due to the extensive cell death and consequent release into the blood stream of the cell contents. The dose of radiation used, although between one-third and one-quarter of the LD_{50}, kills a very high proportion of the small lymphocytes (Trowell, 1952) and of the erythropoietic cells of the bone marrow (Hulse, E. V., 1955, personal communication), as well as some of the intestinal mucosa and of the gonads. The total bulk of cells killed forms perhaps 2 per cent of the body weight, yet histologically twenty four hours after irradiation the sites of cell death are largely free from dead and disintegrating cells. Thus radiation may be thought of as providing a slow intravenous infusion of first class protein with a consequent rise in blood sugar and liver glycogen.

Phenomena, which formerly were explained as due to non-specific stress, have been suggested here to be physiological consequences of cell death, a specific effect of irradiation. With this result in mind, but recognising the insufficiency of the presently available evidence, it is suggested that stress is as meaningful a word as shock. We may think the ideas useful in a broad sense but as soon as mechanisms are investigated in detail it would appear that the non-specific response is really the sum of specific responses. Somatic stress is compounded of specific responses to starvation, dehydration, cell death and so on, just as shock is compounded of specific responses to blood and fluid loss, infection, pain and psychic disturbance. Some of these specific responses may share final common paths, and one of these may be adrenal activity, but when the specific responses are abstracted there may be nothing left of a general syndrome which needs explanation in non-specific terms.

SUMMARY

Whole body irradiation of the rat has been taken as a typical "stress situation". Evidence that irradiation stimulates adrenal activity is shown to have been misinterpreted: in fact the irradiated rat uses cortisone more efficiently than controls. This result suggests (1) the need for stricter criteria of increased adrenal activity, and (2) that other specific consequences of particular stimuli may have been mistaken for non-specific consequences of stress.

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